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UNIVERSITY OF ALBERTA

CARNITINE DEPENDENT FATTY ACID TRANSPORT

BY

© JUDITH SHARRON ATKINS

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF Master of Science

IN

NUTRITION

FACULTY OF HOME ECONOMICS

EDMONTON, ALBERTA

SPRING 1987

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.....Carnitine Dependent Fatty Acid Transport.....

.....

submitted by ....Judith Sharron Atkins.....

in partial fulfilment of the requirements for the degree of ...Master of Science.....

in nutrition.

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ABSTRACT

It was hypothesized that changes in dietary fat level and fatty acid composition would alter liver mitochondrial membrane lipid composition and affect carnitine dependent fatty acid transport into liver mitochondria. In this regard, male Sprague-Dawley rats were fed diets providing 40% or 15% of calories as fat with polyunsaturated to saturated fatty acid ratio of 1.35 or 0.35. High fat diets increased membrane cardiolipin levels by 15%, increased membrane total acidic phospholipid levels by 6% and decreased total neutral phospholipid levels by 4%. High fat diets increased total phospholipid polyunsaturated fatty acid levels by 5%, decreased total phospholipid monounsaturated fatty acid levels by 11% and decreased total phospholipid saturated fatty acid levels by 2%. Diets with high polyunsaturated to saturated fatty acid ratios increased membrane total phospholipid polyunsaturated fatty acid levels by 4%, decreased membrane total phospholipid monounsaturated fatty acid levels by 11% and decreased membrane total phospholipid saturated fatty acid levels by 3%. High fat, low polyunsaturated to saturated fatty acid ratio diets produced the highest hepatic carnitine palmitoylcarnitine translocase and carnitine palmitoyltransferase-1 activities.

Increasing cardiolipin levels, increasing total acidic phospholipid levels, increasing total neutral phospholipid levels and decreasing total neutral to total acidic phospholipid ratios in liver mitochondrial membranes correlated with increasing carnitine palmitoylcarnitine translocase and carnitine palmitoyltransferase-1

functions. Increasing 18:2ω6 and decreasing 20:4ω6 levels in membrane phospholipids correlated with decreasing carnitine palmitoylcarnitine translocase functions. Increasing 18:1ω7 and 18:0 levels in membrane phospholipids correlated with increasing carnitine palmitoylcarnitine translocase functions. Increasing total polyunsaturated, decreasing total monounsaturated and decreasing total saturated fatty acid levels in liver mitochondrial membrane phospholipids correlated with increasing carnitine palmitoyltransferase-1 functions.

Therefore, changing dietary fat level and fatty acid composition altered mitochondrial membrane lipid composition and affected carnitine dependent fatty acid transport into liver mitochondria by altering carnitine palmitoylcarnitine translocase activity.

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INTRODUCTION AND RATIONALE

Postpartum, the neonate must adapt to utilizing fat as a major energy source. Proper functioning of the carnitine shuttle system is required to attain optimum rates of fatty acid oxidation and ketogenesis. Fatty acid oxidation and ketogenesis have a glucose sparing effect. Defects or deficiencies in the carnitine shuttle lead to clinical symptoms similar to those of hypoglycaemia. During the perinatal period activity of the carnitine shuttle increases. Evidence suggests a role for modification of mitochondrial membrane fatty acid composition in carnitine shuttle activation. The carnitine transport shuttle is a membrane bound protein system and, therefore, is likely affected by membrane lipid environment. Previous studies indicate that membrane phospholipid composition may alter activities of membrane bound protein constituents of the carnitine transport shuttle system. It is not known if alterations in membrane fatty acid composition can alter carnitine palmitoylcarnitine translocase or carnitine palmitoyltransferase-1 activities. Thus the present study was designed to examine the effect of dietary fat on changes in liver mitochondrial membrane and the activites of carnitine palmitoylcarnitine translocase and carnitine palmitoyltransferase-1.

REVIEW OF LITERATURE

The carnitine transport shuttle system transports fatty acids across the inner mitochondrial membrane (1-5), stimulating fatty acid oxidation and ketogenesis (1,4,6,7) and maintaining mitochondrial ATP regeneration during lipolysis (3). The carnitine transport system may also provide a mechanism whereby high energy acyl groups produced by β-oxidation are transported out of the mitochondrion (1,7). This system also buffers the acetylation state of mitochondrial CoA from dramatic fluctuations by permitting equilibration of acetyl-CoA/CoA and acetyl-carnitine/carnitine pools (8,9). Activity of the carnitine transport shuttle system markedly increases when increased oxidation of fatty acid occurs, such as at birth (1).

I. CARNITINE TRANSPORT SHUTTLE SYSTEM

The carnitine transport shuttle system is composed of three protein subunits, carnitine acyl transferase I, carnitine acyl transferase II and acyl carnitine translocase. Carnitine acyl transferase I resides on the outer side of the inner mitochondrial membrane, while carnitine acyl transferase II resides on the matrix side (10). Acyl carnitine translocase is a trans-membrane protein spanning the inner mitochondrial membrane. Exact spatial arrangement of the three protein subunits has not been elucidated.

Carnitine acyl transferases I and II catalyze formation and hydrolysis of fatty acyl carnitines (11). Separate carnitine acyl transferases exist with chain length sepcificities that overlap. Six

acyl transferases have been isolated from mitochondria; three inner and three outer (1). These are: acetyl transferase utilizing free fatty acyl groups of two to three carbons in length, octanoyl transferase utilizing free fatty acyl groups of six to ten carbons in length, and palmitoyl transferase utilizing free fatty acyl groups of 14 to 16 carbons in length. Transport of medium chain fatty acyl groups into the mitochondrion can also be carnitine independent (1).

Acyl carnitine translocase activity cannot be ascribed to operations of other mitochondrial transport systems (12). Translocase catalyzes mole to mole exchange of carnitine and acyl carnitines across the inner mitochondrial membrane (1,12,13). Acyl carnitines produced by carnitine acyl transferase I are translocated by the translocase despite the presence of higher concentrations of external free carnitine (13), permitting efficient delivery of acyl carnitines to mitochondrial enzymes of β-oxidation (13) and preventing intra-mitochondrial accumulation of acyl carnitines which could lead to toxic intra-mitochondrial CoA concentrations (9). The translocase appears to remain subsaturated by matrix carnitine concentrations due to low affinity for this substrate (13). It is also probable that translocase remains subsaturated by long chian acyl carnitines in vivo (12).

II. FACTORS AFFECTING FUNCTION

Carnitine palmitoyl transferase I activity in rat liver increases nearly five fold within 24 hours postpartum and peaks within two to three days (14). Processes involved in carnitine dependent fatty acid

transport in humans also appear to be activated at birth. Changes in acetyl transferase and palmitoyl transferase activities during the perinatal period parallel development of fatty acid oxidation in heart, liver and adipose tissue (1,4). Factors responsible for these dramatic increases in activity immediately after birth remain undefined. In this regard, activity of the carnitine shuttle transport system is influenced by carnitine concentration and availability, free fatty acid supply, membrane microenvironment, and hormone action. In neonates it is not known which factor plays a dominant role in activating and controlling carnitine dependent fatty acid transport; however, to date carnitine status is the most studied factor.

(a) Carnitine Status

Carnitine (3-hydroxyl-4-n-trimethylaminobutyrate) is synthesized from lysine, methionine and/or choline (15). Butyrobetaine hydroxylase catalyzes the final rate limiting step in carnitine biosynthesis and is present only in liver, kidney, and brain of humans (1). Butyrobetaine hydroxylase activity is age dependent. In liver of normal infants less than three months of age its activity is less than 12% of adult activity (16). Liver is the primary biosynthetic site of carnitine and releases free and acyl carnitines into circulation to supply other non-synthesizing tissues (1).

Carnitine levels required for optimal activity of the carnitine shuttle transport system and thus optimal fatty acid oxidation and ketogenesis are unknown. Low muscle and liver carnitine levels are associated with low but variable serum carnitine levels and excess

lipid in muscle and other tissues (18). Primary systemic carnitine deficiency is also clinically characterized by recurrent episodes of metabolic encephalopathy, muscle weakness and other symptoms consistent with hypoglycaemia (17,18). Hypoglycaemia can occur during a carnitine deficient state due to limited fatty acid transport or utilization by tissues or impaired hepatic gluconeogenesis which requires energy from fatty acid oxidation (18,54). Many studies have been undertaken to determine important factors affecting carnitine status in the neonate.

Fetuses and neonates are likely incapable of endogenous synthesis of carnitine, due to low activity of butyrobetaine hydroxylase (7,16). In utero, fetal serum and tissue carnitine concentrations are dependent upon maternal carnitine status (7,15) and the rate of placental transfer (8,19). Postpartum neonatal carnitine levels are dependent upon exogenous sources such as mother's milk, formula, and other dietary replacements or supplements.

It has been postulated that carnitine levels in breast milk may reflect the needs of the infant (20). Breast milk total carnitine content increases during the first week postpartum, with the highest concentration occurring during the first two to three days of suckling (6,21). Absolute carnitine content of human milk varies depending on maternal diet and physiological state. During the first 21 days postpartum, milk total (free and acyl) carnitine content varies from 50 to 70 nmol/ml (1,20,22) and falls to approximately 35 nmol/ml 40 to 50 days postpartum (2,20).

Fresh and commercial pasteurized cow's milk contains 190 to 270 nmol/ml and 160 to 200 nmol/ml of carnitine, respectively (20). Cow's

milk contains more carnitine than human milk, but breast fed infants have higher serum carnitine levels than formula fed infants during the first 48 hours of life, suggesting greater bioavailability of carnitine in breast milk than in cow's milk (6).

Soya based formulas contain little or no carnitine (22). Lipid emulsions, such as Intralipid™ and Nutralipid™ contain less than 1 uM of carnitine (25). Both soya based formulas and lipid emulsions produce significantly low levels of plasma carnitine and ketone bodies in neonates (2,22). Small for gestational age and premature infants receiving low or carnitine free total parenteral nutrition for more than 15 days have reduced liver carnitine levels which may impair fatty acid oxidation and ketogenesis (16,23).

Intravenous L-carnitine supplementation in neonates can maintain normal plasma carnitine levels (23,24) whereas unsupplemented infants cannot (23), suggesting that exogenous sources of carnitine are required by the neonate. Studies exist which do not support this theory. In this regard, exogenous carnitine administered to neonates was acylated in large amounts, with no improvement in utilization of infused lipids observed (24). When no carnitine was fed, neonatal blood levels of carnitine remain relatively constant, but low (25), suggesting that some carnitine was supplied endogenously. Liver synthesis in neonates is minimal, therefore endogenous carnitine maybe supplied from storage sites (16,25). In neonates, muscle is the primary site of carnitine storage (17), but no evidence exists to indicate that muscle carnitine buffers plasma carnitine levels. Thus, controversy exists over whether or not carnitine supplementation of the

neonate is required.

Carnitine concentrations in mammalian tissues vary widely depending on the tissues studied and the assay methods. Neonatal liver carnitine content does not correlate with gestational age or body dimensions and are markedly lower than adults (17). Adult liver carnitine levels are achieved by seven months of age apparently due to increased hepatic synthesis and carnitine intake from exogenous sources (15). Plasma concentrations of carnitine are commonly used to determine carnitine status (26); however, as plasma carnitine levels do not necessarily reflect tissue carnitine levels it is an inadequate marker of carnitine status (24,26).

Adequate plasma and tissue carnitine concentrations are important, but sufficient mitochondrial content of carnitine is essential. Previous studies have shown the rate of carnitine-carnitine exchange in mitochondria to be influenced by change in matrix carnitine concentration (13). Increasing carnitine content in the mitochondrial matrix and increasing long chain acyl carnitine concentrations extramitochondrially, increases translocase activity and favours increased flux of fatty acids through mitochondrial β-oxidation (12). Neonatal hepatic mitochondrial matrix carnitine concentration has not been reported. Determination of the carnitine content in the mitochondrial matrix compartment in liver, although at present clinically impractical, may provide the best measure of carnitine status in the neonate.

(b) Free Fatty Acid Supply

Decreased plasma free fatty acid levels decrease uptake and transport of fatty acid into mitochondria by the carnitine transport shuttle system and thus decrease rate of fatty acid oxidation and ketogenesis. In the immediate postnatal period, serum levels of free fatty acids rise dramatically, indicating active lipolysis of adipose tissue (6). After 12 to 24 hours of extra-uterine life the rate of adipose lipolysis falls rapidly (11) as feeding is initiated, ensuring continued free fatty acid supply. Rates of lipolysis in the first few hours of life appear to be regulated by increased catecholamine release, resulting in increased cAMP production and protein kinase activity, thus activating adipose tissue lipoprotein lipase (2,6,27,28). In vitro experiments illustrate that lipolysis is enhanced more by addition of exogenous glucose to increase energy supply than by addition of hormones (11), indicating that lipolysis in neonatal adipose tissues may depend more upon mobilization of glycogen stores than on catecholamine stimulation (29). Insulin and glucagon also play a role in determining free fatty acid supply in the neonate. Insulin stimulates lipogenesis and glucagon stimulates lipolysis (30,31).

(c) Membrane Microenvironment

Membrane properties, such as fluidity, play a significant role in determining receptor, transport and enzymatic activities (32). Fluidity is a descriptive term used to describe the lateral rates of motion of lipids and proteins in a membrane (33). Fluidity of a membrane is

directly influenced by the degree of unsaturation of its constituent lipids (33); increasing the degree of unsaturation increases the fluidity. Membrane associated proteins have also been noted to increase activity with increased membrane lipid unsaturation and increased fluidity. Fluid membranes facilitate function of some integral membrane proteins by offering less resistance to steric movements of polypeptide domains. Essential fatty acids, being polyunsaturated, increase membrane fluidity. Essential fatty acid deficiency has been related to functional lesions of the inner mitochondrial membrane, such as impaired substrate oxidation and partial uncoupling of oxidative phosphorylation (34).

Some membrane proteins require specific lipids for optimal function. Most membrane lipids do not participate directly in the reactions catalysed by intrinsic membrane proteins. Instead, lipids exert their influence by interacting at hydrophobic regions of the protein distinct from the active site. Lipids can, therefore, be considered allosteric effectors of membrane associated enzymes. Some cellular reactions involving membrane-associated enzymes can be regulated in vivo by changing the membrane lipid composition or by altering membrane fluidity (33).

i) Fatty Acid Composition

Lipid composition of fetal tissues is influenced by many factors. For example, maternal diet limits types and amounts of essential fatty acids available in the maternal circulation (35). Differential placental transfer or metabolism of circulating maternal fatty acids

also limits fatty acids available to the fetus (36). Non-essential fatty acids, such as palmitic acid, can be synthesized de novo by the fetus provided an adequate amount of substrate is available (4,35,36). At birth, neonatal adipose tissue is largely composed of saturated (16:0) and mono-saturated (16:1 and 18:1) fatty acids. Palmitic acid (16:0) constitutes less than 50% of the fatty acids, while linoleic acid (18:2), an essential fatty acid, constitutes less than 1% (37); the latter is far below maternal adipose tissue linoleic content (38). Intrauterine liver accretion of essential fatty acids occurs in normally developing infants and provdes a small labile pool (39).

Post partum adipose tissue essential fatty acid content increases 5 weeks before liver essential fatty acid content (39,40,41). Longer chain polyunsaturated fatty acids ($\geq C_{20}$) are mobilized from liver, while 18:2 content increases during this time (39). After 5 weeks, there is an increase in all polyunsaturated fatty acids (39,40). Neonatal adipose tissue composition changes rapidly after birth and reflects dietary fat intake (37). Lack of neonatal hepatic polyunsaturated fatty acid accretion suggests limited endogenous polyunsaturated fatty acid synthesis (39) or immature hepatic polyunsaturated fatty acid uptake and/or utilization mechanisms. Rapid mobilization and relatively slow repletion of longer chain hepatic polyunsaturated fatty acids during the perinatal period may affect hepatic membrane composition, resulting in altered membrane fluidity. Altered membrane fluidity may significantly affect activities of membrane bound hepatic enzymes and/or transport systems.

ii) Phospholipid Composition

Alterations in phospholipid head group composition affect fluidity, and polar interactions in the membrane microenvironment (42). Phospholipid composition of inner mitochondrial membrane and palmitoyl transferase I activity are sequentially modified by D-galactoseamine (42). It is not established how D-galactoseamine modifies membrane phospholipid composition to alter palmitoyl transferase I activity, but depletion of specific phospholipid species seems to be involved (43). In rats, mitochondrial membrane phospholipid content and fatty acid composition in rats is age dependent (44). Neonatal rats have higher phospholipid concentrations than adults. Phospholipid fatty acid composition also differs between the two age groups (44). Rat perinatal mitochondria contain lower levels of cardiolipin than adult rats. Cardiolipin contains large amounts of linoleate. Low cardiolipin levels in inner mitochondrial membranes may contribute to immature functions of mitochondria during the perinatal period by limiting amounts of linoleate in the membrane (44). Current research indicates that cardiolipin is essential for optimal activity of the carnitine transport shuttle system (45).

(d) Hormones

Characteristic hormonal profiles observed at birth may activate proteins of metabolic pathways required for postpartum survival, such as the carnitine transport shuttle system. Catecholamines, insulin, glucagon, growth hormone, thyroxine, and sex hormones all affect carnitine dependent fatty acid transport. These factors act directly

by modifying carnitine acyl transferase and/or acyl carnitine translocase activity or indirectly by increasing free fatty acid and/or carnitine availability.

Carnitine dependent fatty acid transport rates are affected by epinephrine induced inhibition of insulin release (46,47) and activation of lipolysis and decreases in adipose tissue lipoprotein lipase activity (47). Insulin acts to block adrenergic receptors; therefore, suppression of insulin secretion increases the catecholamine effect (27). Administration of norepinephrine to adult rats increases oxygen consumption (3,48), and together with carnitine approximately doubles the increase in oxygen consumption due to norephinephrine alone (48). Carnitine administration, alone, has no effect on oxygen consumption (48). Therefore, norepinephrine likely increases oxygen consumption by increasing availability of free fatty acids to the carnitine transport shuttle system and consequently to mitochondrial oxidation (3,48). Increased lipolysis and decreased adipose tissue lipoprotein lipase activity also increases free fatty acid availability, thus increasing carnitine dependent fatty acid transport.

Insulin and glucagon constitute a bihormonal control system of carnitine dependent fatty acid transport (30). Insulin deficiency at adipose sites causes mobilization of fatty acids (31,49). Insulin suppresses carnitine palmitoyl transferase activity and also inhibits ketone body formation in cultured hepatocytes. By suppressing carnitine palmitoyl transferase activity, insulin may act as a long term regulation mechanism (50). In rats, glucagon increases liver carnitine (1); in humans, glucagon decreases plasma carnitine, likely

due to increased tissue (including liver) uptake (1). Glucagon also increases the carnitine mitochondria content (12), and accelerates fatty acid oxidation in liver by activating carnitine acyl transferases (49) through cAMP (50). Increased glucagon and long chain acyl CoA esters may also increase fatty acid oxidation by decreasing levels of malonyl CoA, a carnitine palmitoyl transferase I inhibitor (6,50). Insulin increases malonyl CoA concentration by stimulating acetyl carboxylase, and thus inhibiting carnitine palmitoyl transferase I activity (1).

Growth hormone and thyroxine may constitute another bihormonal control system. Rats with an over abundance of thyroxine demonstrate increased carnitine palmitoyl transferase activity while rats deficient in thyroxine show decreased carnitine palmitoyl transferase activity (1). It is proposed that the primary action of thyroxine is on mitochondrial membrane fluidity and that all other metabolic effects are secondary, including carnitine transport shuttle activity (32). One action of thyroxine is to decrease microsomal $\Delta 6$-desaturase activity while concomitantly increasing $\Delta 9$-desaturase activity (32), resulting in changes in membrane fluidity and fatty acid composition. Growth hormone may similarly modify mitochondrial membrane fluidity by acting on enzymes of fatty acid desaturation and elongation (32).

Sex hormones also play a role in regulating carnitine plasma and tissue levels (1). No differences in carnitine concentrations between female and male rats have been observed before weaning (51). In rats 22 to 85 days old, males have higher plasma, heart and skeletal muscle carnitine concentrations than do females, but females have slightly

higher liver carnitine concentrations (1,51). It is interesting to note that female rats excrete carnitine at higher rates than do males during this time (51). Human studies also demonstrate male plasma and muscle carnitine levels exceed female levels (51). Preliminary studies by Dr. Peggy Borum indicate that products of ovaries, testes and pituitary gland play a role in controling tissue carnitine concentrations (51).

Nutritional manipulation of carnitine transport shuttle system activity through carnitine concentration, free fatty acid supply and membrane fatty acid composition is likely possible. Postpartum, neonatal carnitine levels increase only upon exogenous supplementation. The degreee of supplementation can theoretically determine plasma carnitine concentration. Increased supplementation makes more carnitine available to the carnitine transport shuttle system. Carnitine requirements are unknown for mammalian species (22), making formulation of nutritional replacements and supplements difficult. Increasing plasma free fatty acid levels by dietary supplementation provides additional substrate and may increase the rate of carnitine dependent fatty acid transport. Diet-induced manipulations of membrane fatty acid composition have successfully been performed in rats. It is possible to increase the unsaturated fatty acid content of rat neonatal membrane by feeding diets high in unsaturated fats. Membrane composition of developing humans can likely be manipulated in the same way. The optimum membrane microenvironment required for maximal carnitine transport shuttle activity is unknown. Dietary manipulation of membrane fatty acid composition may improve substrate utilization in an infant by altering membrane associated protein activity.

SUMMARY

Long chain fatty acid oxidation and ketone body utilization are major metabolic adaptations at birth (4). The neonate must adapt from utilization of a high carbohydrate diet in utero to a high fat diet postpartum. In neonates, plasma levels of ketone bodies increase a few hours after birth with maximum levels occurring by the second to third day of life (52). This increase in ketogenesis parallels increased fatty acid oxidation (53).

One regulator of fatty acid oxidation and ketogenesis at the cellular level is fatty acid transport into mitochondria via the carnitine transport shuttle system located in the inner mitochondrial membrane (27,52). Activity of this system increases at birth. Many factors affect activity and function of the carnitine transport shuttle system in the neonate; these include carnitine status, free fatty acid supply, membrane microenvironment and hormone action. An adequate amount of carnitine must be present for active carnitine dependent transport of fatty acids (30). Carnitine requirements are unknown for mammalian species (22). Therefore, numerical definitions of adequacy do not exist. Postpartum, carnitine levels increase only upon exogenous supplementation. Carnitine deficiency leading to impaired carnitine shuttle system activity is manifested by sub-optimal rates of fatty acid oxidation and ketogenesis (5). Decreased rates of fatty acid oxidation and ketogenesis may lead to symptoms consistent with hypoglycaemia (17,18).

Free fatty acids are co-factors with carnitine in the carnitine shuttle system. In the immediate postnatal period, serum levels of free fatty acids rise dramatically, ensuring adequate supply for fatty acid oxidation and ketogenesis. Free fatty acids may also be provided through dietary intake and nutritional replacements and supplements.

During the perinatal period, fatty acid composition of the neonate changes from a low unsaturated fatty acid content to a higher unsaturated fatty acid content. This shift in fatty acid saturation is mediated by diet and the characteristic hormonal profile seen at birth. The neonate's capability to produce polyunsaturated fatty acids is limited, and polyunsaturated fatty acids must therefore be exogenously provided. Proper membrane fluidity is required for optimal activity of many membrane-associated enzyme and transport systems; membrane fluidity is affected by the content of unsaturated fatty acids. Specific lipids are also required by some membrane proteins for optimal activity. The membrane microenvironment required for optimal carnitine transport shuttle system activity is unknown, except that cardiolipin seems to be required. Dietary fat intake has been proven to modify membrane composition in neonatal rats and may be useful in modifying human membrane lipid composition.

The characteristic hormonal profile at birth may play a role in activation of many metabolic systems, including the carnitine transport shuttle system. Hormones affect carnitine transport shuttle system activity directly by modifying protein subunit function and indirectly by modifying membrane fluidity.

Gluconeogenesis is the major determinant of hypoglycaemia in

neonates utilizing fat as a major energy source. Gluconeogenesis requires energy in the form of ATP and NADH supplied by -oxidation of fatty acids. Decreased fatty acid oxidation due to to decreased fatty acid transport into mitochondria by the carnitine transport shuttle system could, therefore, lead to hypoglycaemia in a neonate. Research into the roles hormones and membrane microenvironment play in the activation and activity of the carnitine transport shuttle system during the perinatal period may be important in determining formulation of nutritional replacements and supplements, especially in the treatment of neonates predisposed to hypoglycemia.

HYPOTHESIS

It is generally hypothesized that changes in dietary fat level and fatty acid composition alter liver mitochondrial membrane lipid composition and affects carnitine dependent fatty acid transport into liver mitochondria. It is specifically hypothesized that:

1) increasing unsaturated fatty acid content of dietary fat increases unsaturated fatty acid content of liver mitochondrial membrane phospholipids.
2) increasing unsaturated fatty acid content of dietary fat increases liver mitochondrial membrane levels of major unsaturated fatty acid containing phospholipids, such as cardiolipin.
3) increasing liver mitochondrial membrane levels of major phospholipids, such as cardiolipin, phosphatidylcholine and phosphatidylethanolamine, increases activity of carnitine palmitoyltransferase-1 and carnitine palmitoylcarnitine translocase.
4) increasing unsaturated fatty acid content of liver mitochondrial membrane phospholipids increases activity of carnitine palmitoyltransferase-1 and carnitinepalmitoyl translocase.

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INTRODUCTION

The carnitine transport shuttle system, which transports long chain fatty acids into mitochondria for β-oxidation or ketogenesis, is comprised of carnitine palmitoyltransferase-1, carnitine palmitoyltransferase-2 and carnitine palmitoylcarnitine translocase (1,2). Carnitine palmitoylcarnitine translocase is an integral membrane protein spanning the inner mitochondrial membrane and carnitine palmitoyltransferase-1 and carnitine palmitoyltransferase-2 are peripheral membrane proteins located on the outer and inner faces of the inner mitochondrial membrane, respectively (2). Previous studies on other membrane proteins have shown that membrane lipid composition affects activity of membrane proteins (3-11). Previous studies have also shown that membrane lipid composition and activity of membrane proteins can be altered by dietary fat composition (12-15). Evidence exists to support the hypothesis that membrane lipid composition also affects carnitine palmitoyltransferase-1 and carnitine palmitoylcarnitine translocase activities. Carnitine palmitoyltransferase activity has been shown to increase with membrane incorporation of cardiolipin and/or phosphatidylcholine (16-19). Carnitine palmitoylcarnitine translocase activity had also been shown to increase with membrane incorporation of cardiolipin and/or phosphatidylcholine (20,21). The effects of membrane fatty acid composition on carnitine palmitoyltransferase and carnitine palmitoylcarnitine translocase activities has not been reported. The objective of this study was to determine whether altering dietary fat

levels and dietary fatty acid composition will effect carnitine palmitoylcarnitine translocase and carnitine palmitoyltransferase-1 activities by modulating membrane phospholipid and phospholipid fatty acid composition.

MATERIALS AND METHODS

I. ANIMALS AND DIETS

Weanling male Sprague-Dawley rats weighing 46.7 ± 4.6 g were obtained from University of Alberta Laboratory Animal Services and were fed semi-purified diets (table 1) for at least fourteen days. Diets and water were supplied ad libitum to rats housed individually in a temperature (22°C) and light controlled room providing twelve hour light and dark periods. Rats were randomized into four groups and fed one of four diets (n=12). The four diet treatments were: high fat, high polyunsaturated to saturated fatty acid ratio; low fat, high polyunsaturated to saturated fatty acid ratio; high fat, low polyunsaturated to saturated fatty acid ratio and low fat, low polyunsaturated to saturated fatty acid ratio. High fat diets provided 40% of calories as fat and 7.2% of calories as linoleic acid, while low fat diets provided 15% of calories as fat and 3.3% of calories as linoleic acid. Mixtures of soyabean oil and beef tallow were used to provide the dietary fat source. The polyunsaturated to saturated fatty acid ratios of high and low polyunsaturated to saturated fatty acid ratio diets was 1.35 and 0.35, respectively (table 2). Nutrient densities for non-fat components were similar between the four diets on a per calorie basis. Cholesterol levels of the four diets were equalized to the cholesterol content of the high fat, low polyunsaturated to saturated fatty acid ratio diet by addition of cholesterol to the three diets lower in cholesterol content. Diet treatments were initiated upon arrival of the rats and continued for a

Table 1. Composition of Experimental Diets

Ingredient	High Fat Diet		Low Fat Diet	
	High P/S	Low P/S	High P/S	Low P/S
	g			
High protein casein	274.1	274.1	237.0	237.5
Cornstarch	188.7	188.7	406.1	407.8
Glucose	211.2	211.2	182.6	183.0
Fat mixture	202.0	200.0	65.5	65.0
Nonnutritive cellulose[2]	48.9	50.8	44.3	42.6
Vitamin mix[3]	10.3	10.3	8.9	9.0
Mineral mix[3]	51.7	51.9	44.7	44.8
Choline	4.0	4.0	3.4	3.5
Inositol	6.3	6.3	5.4	5.4
L-Methionine	2.7	2.7	2.3	2.3
Cholesterol (mg)	107.0	-----	158.0	122.0

[1]P/S, polyunsaturated to saturated fatty acid ratio.

[2]AOAC vitamin mix (Teklad Test Diets, Madison, WI) provided the following per kilogram of complete diet: vitamin A, 20,000 IU; vitamin D, 2000 IU; vitamin E, 100 mg; menadione, 5 mg; thiamin-HCl, 5 mg; riboflavin, 8 mg; pyridoxine-HCl, 40 mg; niacin, 40 mg; pantothenic acid, 40 mg; choline, 2 g; myo-inositol, 100 mg; p-aminobenzoic acid, 100 mg; biotin, 0.4 mg; folic acid, 2 mg; and vitamin B_{12}, 30 mg.

[3]Bernhart-Tomarelli mineral mix (General Biochemicals, Chagrin Falls, OH) provided the following per kilogram of complete diet: Ca, 11.246 g; Cl, 0.928 g; Cu, 8.112 mg; I, 0.2625 mg; Fe, 46.5 mg; Mg, 754 mg; Mn, 58.0 mg; P, 9.3204 g; K, 3.344 g; Na, 999.5 mg; S, 6.2560 g; Zn, 21.4 mg.

Table 2. Fatty Acid Composition of Diets[1]

Fatty Acid (%w/w)	High Fat Diet		Low Fat	
	P/S=1.35	P/S=0.35	P/S=1.35	P/S=0.35
16:0	22.18	31.84	24.79	34.02
16:1	1.74	4.06	1.65	4.38
18:0	6.47	12.82	5.68	11.20
18:1ω9	22.18	27.54	21.10	26.75
18:1ω7	1.52	1.96	1.57	1.93
18:2ω6	36.86	13.63	36.58	14.84
18:3ω3	6.05	2.29	6.05	2.50
Total saturated	30.77	49.68	32.70	50.37
Total Monounsaturated	23.90	29.96	22.67	29.19
Total Polyunsaturated	43.77	17.61	42.66	17.69

[1]High fat diets (40% of calories as fat) and low fat diets (15% of calories as fat) were fed. The polyunsaturated to saturated fatty acid (p/s) ratio was adjusted by beef tallow for soyabean oil. The basic differences and appropriate comparisons between diet treatments are described in the Materials and Methods section.

minimum of 14 days or a maximum of 17 days. Rats, with a final body weight of 129.1 ± 15.4 g, were sacrificed by decapitation and their livers quickly removed and placed on ice until mitochondrial isolation. There was no difference in final rat body weights between dietary treatments.

II. MITOCHONDRIAL ISOLATION

Mitochondria were isolated from rat livers using a procedure modified from Beiber et al (22). The livers were minced, washed three times with chilled isolation medium containing: 220 mM mannitol, 70 mM sucrose, 2 mM HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic Acid, pH 7.4), and 1 mM EDTA (Ethylenediaminetetraacetic Acid). Minced livers were homogenized using fresh isolation medium in a T-line Laboratory Stirrer and a teflon and glass homogenizer to obtain a 23% (w/v) homogenate. The homogenate was centrifuged at 4°C (600g X 15 min.) and mitochondria pelleted from the resulting supernatant (7,700g X 15 min.). The second supernatant was discarded and the pellet resuspended in fresh isolation medium. The mitochondrial pellet was washed twice more (7,700g X 15 min.). The final pellet was resuspended in 1 ml of isolation medium. Mitochondrial respiratory control and ADP:O values were calculated (23,24). The remaining mitochondrial suspension was increased to a volume of 6 mls using 10 nM phosphate buffer (pH 7.0) containing 1 mM dithiothreitol and 1 ml aliquots were stored frozen at -70°C for later protein and lipid analysis. Protein was measured by a modified Lowry procedure (25).

III. CARNITINE PALMITOYLTRANSFERASE-1 ASSAY

The rate of formation of palmitoylcarnitine from palmitoyl-CoA and carnitine by carnitine palmitoyltransferase-1 was measured using a modified procedure of McGarry et al (26). Reactions were carried out at 30°C in borosilicate glass test tubes (13mmX100mm) in a shaking waterbath. The standard incubation mixture, in a volume if 0.8 ml, contained: 130 mM tris/HCl (pH 7.4); 19 mM KCl; 2.5 mM KCN; 5.0 mM ATP; 5.0 mM $MgCl_2$; 40 µg rotenone; 10 mg bovine serum albumin; 0.31 mM reduced glutathione; 2.5 mM sodium tetrathionate; 40 µM palmitoyl-CoA; 0.05 µCi DL-[methyl-^{14}C]-carnitine; 40 µM to 400 µM L-carnitine. The reaction mixture was preincubated for 4 minutes at 30°C. Reactions were initiated by addition of 10 µl of mitochondrial suspension (0.1 to 0.3 mg protein) and incubated for 4 minutes. The reaction was terminated by addition of 1 ml of 1.2 N HCl. Termination solution was added prior to the addition of mitochondria in control reaction mixtures. The labelled palmitoylcarnitine formed was extracted by addition of 1.0 ml butanol. The tubes were vortexed (approximately 30 seconds), centrifuged to separate the phases, and 0.5 ml of the butanol layer was removed and added to another tube (13mmX100mm) containing 0.1 ml water and 0.5 ml butanol saturated with water. After vortexing and centrifuging, 0.2 ml of the butanol layer was counted with 5.0 mls aquasol in a Beckman LS 5801 scintillation counter.

The Beckman scintillation counter utilized a quench compensation program based on H numbers to calculate DPM's with a counting efficiency of approximately 94.6%. The automatic quench compensation program was calibrated using Beckman ^{14}C quench standards.

IV. CARNITINE PALMITOYLCARNITINE TRANSLOCASE ASSAY

[1-^{14}C]-palmitoylcarnitine transport into rat liver mitochondria by carnitine palmitoylcarnitine translocase was assayed using a modified procedure of Murthy and Pande (1) to measure production of citric acid cycle intermediates derived from the acyl portion of transported palmitoylcarnitine. Reactions were carried out at approximately 0°C in borosilicate glass test tubes (13mmX100mm) in a shaking ice water bath. The standard incubation mixture, in a volume of 0.05 ml, contained: 250 mM mannitol; 25 mM HEPES (pH 7.4); 0.05 mM EDTA; 3 mM ADP; 1 mM malate; 5 mM potassium phosphate, 0.025 µCi 1-^{14}C-palmitoyl-carnitine; 1 µM to 10 µM palmitoylcarnitine. The reaction mixture was preincubated for 2 minutes at 0°C. Reactions were initiated by the addition of 10 µl of mitochondrial suspension (0.1 to 0.3 mg protein) and incubated for 2 minutes. Palmitoylcarnitine transport into mitochondria was terminated by the addition of 200 µl of 2.5 mM merslayl; 250 mM mannitol and 50 mM Tris/HCl (pH 7.4) solution. Termination solution was added prior to the addition of mitochondria in control reaction mixtures. The tubes were incubated for a further 10 minutes at 0°C to ensure conversion of imported 1-^{14}C-palmitoylcarnitine to ^{14}C-anions. Chloroform (200 µl) was added and tubes were vortexed vigorously to denature mitochondrial proteins. After centrifugation, 100 µl of the aqueous layer was applied to pipette columns (0.5cmX2.5cm) containing AG50W-X8 ion exchage resin (200 to 400 mesh) in water and eluted with two washes of 0.5 ml water. The eluent was counted with 5 mls aquasol in a Beckman LS 5801 scintillation counter as above.

V. LIPID EXTRACTION

Mitochondrial membrane lipids were extracted using a modified Folch procedure (27). Methanol (1 ml) containing ethoxyquin as antioxidant was added to 1 ml of the stored mitochondrial suspension contained in small methylation vials. The vials were capped, vortexed, extracted with 2 ml chloroform, vortexed, and placed on ice. Protein was separated from the extraction mixture by centrifugation. The extraction mixture was run through pipettes containg glass wool and collected in small methylation tubes. The remaining protein precipitate was re-extracted using the above procedure. Three mls of chloroform:methanol (7:1, v/v) saturated with 28% (w/v) aqueous ammonium was added to the doublely extracted protein precipitate followed by the addition of 0.92 mls of methanol, 2.4 mls of 0.1 M KCl and vortexing (28). The extraction mixture was run through pipettes containing glass wool and collected in small methylation tubes. The above three extraction mixtures were pooled and the resultant chlorform layer was removed and evaporated to dryness under vaccuum. The lipid residue was resuspended in 1.0 ml of chloroform: methanol (19:1 v/v) and stored in small vials at -70°C for later analysis.

VI. SEPARATION OF LIPIDS

Phospholipids were separated using a modified method of Touchstone et al (29). High performance thin layer chromatography plates (Whatman, HP-K high performance silica gel plates, 10 cm X 10 cm) were activated by heating one hour at 140°C. Plates were then spotted with

150 µl of the lipid extract and developed in small development tanks using chloroform: methanol: 2-propanol: 0.25%, (w/v), potassium chloride: triethylamine (30:9:25:6:18, by volume). Plates were air dried and sprayed with 0.03% (w/v) 2'-7'-dichlorofluorescene in 0.01 M sodium hydroxide and phospholipid spots visualized under U.V. light. Spots were scraped into methylation vials for fatty acid analysis or phosphorous determinations. Cardiolipin spots were not clearly resolved from neutral lipid spots. Therefore, for accurate cardiolipin fatty acid analysis, the cardiolipin-neutral lipid spots were eluted from the silica using chloroform: methanol (2:1,v/v). The eluents were evaporated to dryness, resuspended in cholorform:methanol (19:1,v/v), spotted on 1" X 3" G-plates (Whatman, MK6F silica gel plates previously activated for one hour at 140°C) and developed in petrolum ether: diethyl ether: formic acid (15:10:0.4,v/v/v). Plates were air dried and cardiolipin spots visualized as above. The resolved cardiolipin spots were scraped into methylation vials for fatty acid analysis.

VII. FATTY ACID ANALYSIS

Fatty acids were methylated using a modified boron-trifluoride technique of Morrison and Smith (30). Two mls of distilled hexane and 1.5 mls of boron-trifluoride-methanol reagent were added to methylation tubes containing scraped phospholipid spots. Tubes were tightly capped and samples heated at 95°C to 105°C. Scraped phospholipid spots were heated for one hour while scraped sphingomyelin spots were heated for one and one half hours. Samples were cooled and 1.0 ml of water was added followed by capping, vortexing and incubated for 25 minutes at

room temperature. The upper hexane phase was removed and evaporated to dryness in micro-vials. Vials were capped with teflon lined lids and stored at -70°C until GC analysis. Fatty methyl esters were separated by gas chromatography (Varian model 6000) and quantified using flame ionization detectors (3). Chromatography was performed using a bonded silica phase capilliary column (25 m X 0.22 mm I.D.) coated with BP20 vitreous silica (S.G.E. Pty, Ltd., Australia). Helium was used as the carrier gas at a column flow rate of 1.08 ml/min. and inlet pressure of 540 kPa. The inlet splitter was set at 8 to 1. Injector and detector temperatures were maintained at 250°C. Column temperature was programmed to increase at a rate of 20°C per min. from 150°C to 190°C and then increase at a rate of 3°C per min. from 190°C to 220°C. Authentic standard mixtures of fatty acid methyl esters were used to identify liver mitochondrial membrane phospholipid fatty acid methyl esters. Identified fatty acids were comprised of the ω-3 series C18:3, C20:3, C20:4, C20:5, C22:6; the ω-6 series C18:2, C18:3, C20:2, C20:3, C20:4, C22:4, C22:5; the ω-7 series C16:1, C18:1; the ω-9 series C18:1, C20:1, C20:3 fatty acids and C14:0, C15:0, C16:0, C18:0, C19:0, C24:0 saturated fatty acids. Peak areas and weight percent fatty acid composition were computed by a chromatography data system (Vista 402, Varian Canada, Ltd.).

VIII. PHOSPHORUS DETERMINATION

Phospholipids were quantitated using a modified version of Menzel and Corwin's colourimetric phosphorus determination (31). Phospholipids and potassium phosphate standards were digested by

autoclaving (120°C X 30 min.) in 0.03 M potassium persulfate solution. Spectrophotometric determination of Pi in cooled samples was accomplished using 100 µl of chromogenic solution (5.89 mM ammonium molybdate; 0.50 mM potassium antimony tartarate; 0.56 M sulfuric acid and 0.06 M ascorbic acid) per ml of potassium persulfate solution. Samples were vortexed and incubated for 30 min. at room temperature. Absorbance was read at 885 nm.

IX. STATISTICAL ANALYSIS

Carnitine palmitoylcarnitine translocase, carnitine palmitoyltransferase-1 activity and phosphorus determinations were assayed in duplicate and then averaged. Effect of diet treatment on lipid composition and enzyme activity was examined by two way analysis of variance procedures followed by Student-Neuman-Keul multiple range tests if an effect of diet was found (32). Relationships between lipid composition and enzyme activity were examined using scatterplots and linear regression of activity versus lipid composition (32).

RESULTS

I. EFFECT OF DIET TREATMENT ON LIVER MITOCHONDRIAL MEMBRANE PHOSPHOLIPID CONTENT

Major phospholipid constituents of liver mitochondrial membranes were phosphatidylcholine, phosphatidylethanolamine and cardiolipin, comprising greater than 23%, 24%, and 20% (w/w), respectively, of total membrane phospholipids (table 3). Minor phospholipid constituents of liver mitochondrial membranes were phosphatidylinositol, sphingomyelin, phosphatidylserine, and phosphatidylglycerol, comprising greater than 10%, 8%, 7% and 6% (w/w), respectively, of total mitochondrial membrane phospholipids. Cardiolipin levels were elevated by 15%, ($p<0.02$), in liver mitochondrial membranes of rats fed diets containing 40% of calories as fat compared to rats fed diets containing 15% of calories as fat. Total acidic phospholipid (cardiolipin, phosphatidylinositol, phosphatidylserine and phosphatidylglycerol) levels were elevated by 6%, ($p<0.03$), and paralleled cardiolipin distribution (table 4). Total neutral phospholipid (phosphatidylcholine, phosphatidylethanolamine and sphingomyelin) levels of liver mitochondrial membranes of rats fed low fat diets were 4.3% higher than in liver mitochondrial membranes of rats fed high fat diets ($p<0.05$). Total neutral to total acidic phospholipid ratios were 9.4% lower in liver mitochondrial membranes of rats fed high fat diets than in liver mitochondrial membranes of rats fed low fat diets ($p<0.03$). No effect of dietary fat or fatty acid levels on phosphatidylcholine to phosphatidylethanolamine ratio was observed (table 4). No significant differences in liver mitochondrial

Table 3. Effect of High versus Low Fat Diets and High versus Low Dietary Polyunsaturated to Saturated Fatty Acid Ratios on Liver Mitochondrial Membrane Phospholipid Composition

Phospholipid	Phospholipid content (ug/mg protein)[1,2]				Two-way Analysis of Variance	
	High Fat Diet		Low Fat Diet			
	P/S[3]=1.35	P/S=0.35	P/S=1.35	P/S=0.35	Fat	P/S
Phosphatidyl-choline	54.0±14.3	60.0±16.6	50.7±13.8	53.0±19.2	NS[4]	NS
Phosphatidyl-ethanolamine	57.2±19.9	61.0±15.6	57.9±13.6	57.0±11.0	NS	NS
Cardiolipin	51.7±10.3	50.2±10.6	43.7±10.0	43.0±11.1	p<0.02	NS
Phosphatidyl-inositol	25.3± 5.8	28.8±10.9	26.0± 6.5	22.8± 4.6	NS	NS
Sphingomyelin	19.2± 6.0	19.8± 6.1	18.3± 3.2	17.9± 4.7	NS	NS
Phosphatidyl-serine	15.5± 3.3	18.9± 8.3	16.4± 5.3	16.6± 8.2	NS	NS
Phosphatidyl-glycerol	13.1± 4.0	15.7± 6.7	14.5± 8.5	11.6± 5.8	NS	NS
total phospholipid	234.2±35.3	253.8±58.2	230.0±41.4	223.7±50.0	NS	NS

[1] Values are mean ± standard deviation, n=12.
[2] No two groups were significantly different according to Student-Newman-Keuls' Multiple Range Test (p<0.05).
[3] P/S, polyunsaturated to saturated fatty acid ratio.
[4] NS, not significant.

Table 4. Effect of High versus Low Fat Diets and High versus Low Dietary Polyunsaturated to Saturated Fatty Acid Ratios on Liver Mitochondrial Membrane Phospholipid Polar Lipid Composition

Phopholipid Group	Phospholipid Content (% Total Phospholipids)[1,2]				Two-way Analysis of Variance	
	High Fat Diet		Low Fat Diet			
	P/S[3]=1.35	P/S=0.35	P/S=1.35	P/S=0.35	Fat	P/S
Acidic Phospholipids	45.0±3.2	45.4±5.3	42.2±3.4	43.0±3.2	p<0.03	NS[4]
Neutral Phospholipids	55.3±3.8	55.9±5.3	58.5±4.1	57.7±4.0	p<0.05	NS
Neutral/ Acidic Phospholipids	1.24±0.14	1.26±0.25	1.40±0.20	1.36±0.19	p<0.03	NS
Phosphatidyl-choline/ Phosphatidyl-ethanolamine	0.96±0.23	1.01±0.23	0.90±0.20	0.95±0.34	NS	NS

[1] Values are mean ± standard deviation, n=12.
[2] No two groups were significantly different according to Student-Newman-Keuls' Multiple Range Test ($p<0.05$).
[3] P/S, polyunsaturated to saturated fatty acid ratio.
[4] NS, not significant.

membrane total phospholipid levels between dietary treatments were observed (table 3).

II. EFFECT OF DIET ON LIVER MITOCHONDRIAL MEMBRANE PHOSPHOLIPID FATTY ACID COMPOSITION

In the present study, major polyunsaturated, monounsaturated and saturated fatty acid levels were determined in liver mitochondrial membrane phospholipids of rats fed diets providing 40% or 15% of calories as fat with polyunsaturated to saturated fatty acid ratio of 1.35 or 0.35.

(a) Phosphatidylcholine

Major fatty acids found comprising phosphatidylcholine were 18:2ω6, 20:4ω6, 22:6ω3 and saturated fatty acids (table 5). The above fatty acids represented greater than 9%, 26%, 7%, and 45% (w/w), respectively, of mitochondrial membrane phosphatidylcholine fatty acids. Diets containing 40% of calories as fat increased phosphatidylcholine levels of 18:0 by 15% ($p<0.001$) and 20:4ω6 by 13% ($p<0.001$). High fat diets decreased phosphatidylcholine levels of 16:0 by 22% ($p<0.001$) and 18:1 by 25% ($p<0.001$). Phosphatidylcholine total monounsaturated fatty acid levels were lower by 29% ($p<0.001$) in rats fed high fat diets. Phosphatidylcholine levels of total polyunsaturated ω3 and ω6 fatty acids, the sum of 18:2ω6 and 20:4ω6 levels and polyunsaturated to saturated fatty acid ratios were increased by 6% ($p<0.001$), 9% ($p<0.001$) and 6% ($p<0.003$), respectively, in rats fed high fat diets. Total saturated fatty acid levels of

Table 5. Effect of High versus Low Fat Diets and High versus Low Dietary Polyunsaturated to Saturated Fatty Acid Ratio on Liver Mitochondrial Membrane Phosphatidylcholine Fatty Acid Composition

Fatty Acid	Fatty Acid Composition, (%w/w)[1,2]				Two-way Analysis of Variance (p<)		
	High Fat Diet		Low Fat Diet				
	P/S[3]=1.35	P/S=0.35	P/S=1.35	P/S=0.35	Fat	P/S	Int.[4]
16:0	15.6±0.80[a]	15.8±0.98[a]	19.8±1.52[b]	20.6±1.60[b]	0.0001	NS[3]	NS
18:0	29.6±1.19[a]	29.0±1.17[a]	24.2±2.23[b]	25.6±2.36[b]	0.0001	NS	NS
18:1ω9	3.8±0.63[a]	4.8±0.69[b]	4.5±0.58[ab]	6.5±1.15[c]	0.0001	0.0001	0.03
18:1ω7	1.8±0.12[a]	1.8±0.19[a]	2.5±0.43[b]	2.8±0.38[c]	0.0001	NS	NS
18:2ω6	9.7±1.05	9.1±1.69	10.2±2.06	8.9±1.42	NS	0.04	NS
20:4ω6	28.8±1.98[a]	27.2±1.22[a]	25.3±2.39[b]	23.6±2.79[b]	0.0001	0.01	NS
22:6ω3	7.4±0.84	7.5±0.63	7.2±0.72	7.0±0.73	NS	NS	NS
Total SFA[3]	45.7±1.25[ab]	45.4±1.33[ab]	44.6±2.55[a]	46.9±2.14[b]	NS	NS	0.02
Total MUFA[3]	6.4±0.92[a]	7.7±0.97[b]	8.7±1.18[b]	11.3±1.81[c]	0.0001	0.0001	NS
Total PUFA[3]	49.0±1.82[a]	47.7±2.48[ab]	46.9±1.08[b]	44.3±1.84[c]	0.0001	0.001	NS
18:2ω6+ 20:4ω6	38.5±1.78[a]	36.3±1.13[b]	35.5±1.22[b]	32.5±2.04[c]	0.0001	0.0001	NS
P/S	1.1±0.05[a]	1.1±0.09[a]	1.1±0.09[a]	1.0±0.04[b]	0.003	0.002	0.02

[1] Values are mean ± standard deviation of 12 individual animals for each dietary treatment.

[2] Values, within rows, without commom superscripts are significantly different according to Student-Newman-Keuls' Multiple Range Test, (p<.05).

[3] Abbreviations used: P/S = polyunsaturated to saturated fatty acid ratio; NS = no significance; SFA = total saturated fatty acids; MUFA = total monounsaturated fatty acids; PUFA = total polyunsaturated fatty acids.

[4] Int., interaction between effects of dietary fat level and polyunsaturated to saturated fatty acid ratio.

phosphatidylcholine were not altered by dietary fat content.

Polyunsaturated to saturated fatty acid ratio of dietary fat had no effect on 16:0, 18:0, 18:1 7 and total saturated fatty acid content of phosphatidylcholine. Diets with a polyunsaturated to saturated fatty acid ratio of 1.35 increased phosphatidylcholine content of 18:2ω6 by 10% ($p<0.04$) and 20:4ω6 by 6% ($p<0.01$), but reduced phosphatidylcholine 18:1 ω9 content by 27% ($p<0.001$). Phosphatidylcholine total monounsaturated fatty acid levels decreased by 20% ($p<0.001$), in rats fed diets with polyunsaturated to saturated fatty acid ratio of 1.35. Total polyunsaturated ω3 and ω6 fatty acid, the sum of 18:2ω6 and 20:4ω6 levels, and polyunsaturated to saturated fatty acid ratio levels increased by 4% ($p<0.001$), 7% ($p<0.001$), and 6% ($p<0.002$), respectively, in mitochondrial membrane phosphatidylcholine of rats fed diets with polyunsaturated to saturated fatty acid ratio of 1.35.

(b) Phosphatidylethanolamine

Phosphatidylethanolamine was high in 20:4ω6, 22:6ω3 and saturated fatty acids (table 6). 20:4ω6 comprised greater than 28% (w/w) of total phosphatidylethanolamine fatty acids, 22:6ω3 comprised greater than 11% (w/w), and saturated fatty acids comprised greater than 49% (w/w) of total phosphatidylethanolamine fatty acids. Diets providing 40% of calories as fat increased phosphatidylethanolamine levels of 18:0 by 9% ($p<0.002$), and 20:4ω6 by 6% ($p<0.003$). High fat diets lowered phosphatidylethanolamine levels of 16:0 by 24% ($p<0.001$), and 18:1 ω7 by 23% ($p<0.001$). Phosphatidylethanolamine total

Table 6. Effect of High versus Low Fat Diets and High versus Low Dietary Polyunsaturated to Saturated Fatty Acid Ratio on Liver Mitochondrial Membrane Phosphatidylethanolamine Fatty Acid Composition

Fatty Acid	Fatty Acid Composition, (%w/w)[1,2]				Two-way Analysis of Variance (p<)		
	High Fat Diet		Low Fat Diet				
	P/S[3]=1.35	P/S=0.35	P/S=1.35	P/S=0.35	Fat	P/S	Int.[4]
16:0	12.3±1.52[a]	13.8±1.52[b]	17.3±1.46[c]	17.0±0.88[c]	0.0001	NS[3]	0.03
18:0	34.4±4.75[ab]	35.5±2.99[a]	31.6±2.87[b]	31.7±2.54[b]	0.002	NS	NS
18:1ω9	2.5±0.37	2.6±0.28	2.3±0.45	2.5±0.37	NS	NS	NS
18:1ω7	1.3±0.21[a]	1.1±0.11[a]	1.5±0.25[b]	1.6±0.27[b]	0.0001	NS	NS
18:2ω6	2.8±0.60	2.6±0.74	2.7±0.55	2.4±1.01	NS	NS	NS
20:4ω6	30.2±1.40[a]	29.6±2.70[ab]	28.5±1.67[ab]	27.9±1.31[b]	0.003	NS	NS
22:6ω3	11.4±0.78	11.5±1.06	11.3±1.13	11.6±0.99	NS	NS	NS
Total SFA[3]	47.3±2.84	49.0±3.26	49.5±3.26	49.2±2.02	NS	NS	NS
Total MUFA[3]	4.7±0.77	4.5±0.41	-4.7±0.76	5.2±0.67	NS	NS	NS
Total PUFA[3]	48.0±1.72	46.7±3.10	45.8±2.92	45.7±1.87	0.04	NS	NS
18:2ω6+ 20:4ω6	32.8±1.44[a]	32.1±2.35[ab]	31.2±1.72[ab]	30.5±1.12[b]	0.001	NS	NS
P/S	1.0±0.11	1.0±0.10	0.9±0.12	0.9±0.08	NS	NS	NS

[1] Values are mean ± standard deviation of 12 individual animals for each dietary treatment.
[2] Values, within rows, without common superscripts are significantly different according to Student-Newman-Keuls' Multiple Range Test, ($p<0.05$).
[3] Abbreviations used: P/S = polyunsaturated to saturated fatty acid ratio; NS = no significance; SFA = total saturated fatty acids; MUFA = total monounsaturated fatty acids; PUFA = total polyunsaturated fatty acids.
[4] Int., interaction between effects of dietary fat level and polyunsaturated to saturated fatty acid ratio.

polyunsaturated ω3 and ω6 and the sum of 18:2ω6 and 20:4ω6 levels were elevated by 3% ($p<0.04$) and 5% ($p<0.001$), respectively, in rats fed high fat diets. Levels of 18:1ω9, 18:2ω6, total saturated fatty acid, total monounsaturated fatty acid and polyunsaturated to saturated fatty acid ratios in phosphatidylethanolamine remained unchanged by dietary fat levels. Dietary polyunsaturated to saturated fatty acid ratio had no significant effect on fatty acid composition of phosphatidylethanolamine.

(c) Cardiolipin

Major fatty acids found comprising cardiolipin were 18:1, 18:2ω6 and much lower levels of saturated fatty acids than in phosphatidylcholine or phosphatidylethanolamine (table 7). These fatty acids comprised greater than 22%, 55% and 10% (w/w), respectively, of the fatty acids in liver mitochondrial membrane cardiolipin. Diets providing 40% of calories as fat increased cardiolipin levels of 18:2ω6 by 7% ($p<0.009$). Levels of 18:1 were lowered by 9% ($p<0.01$) in cardiolipin for rats fed high fat diets. 16:0, 18:0 and total saturated fatty acid levels of cardiolipin were unchanged by dietary fat content.

Diets with polyunsaturated to saturated fatty acid ratio of 1.35 increased 18:2ω6 content of cardiolipin by 9% ($p<0.001$), but reduced 18:1ω7 content by 5% ($p<0.001$). Total monounsaturated fatty acid levels decreased by 10% ($p<0.002$) in cardiolipin for rats fed diets with polyunsaturated to saturated fatty acid ratios of 1.35. Levels of total polyunsaturated ω3 and ω6 fatty acids, the sum of 18:2ω6 and

Table 7. Effect of High versus Low Fat Diets and High versus Low Dietary Polyunsaturated to Saturated Fatty Acid Ratio on Liver Mitochondrial Membrane Cardiolipin Fatty Acid Composition

Fatty Acid	Fatty Acid Composition, (%w/w)[1,2]				Two-way Analysis of Variance (p<)		
	High Fat Diet		Low Fat Diet				
	P/S[3]=1.35	P/S=0.35	P/S=1.35	P/S=0.35	Fat	P/S	Int.[4]
16:0	6.1±1.85	5.9±1.32	6.0±0.99	7.0±1.52	NS[3]	NS	NS
18:0	2.4±0.84	2.2±0.43	2.3±0.44	2.8±0.60	NS	NS	NS
18:1ω9	7.2±1.37^{a}	8.2±1.32^{a}	7.9±1.48^{a}	9.9±1.48^{b}	0.01	0.001	NS
18:1ω7	13.7±1.66^{a}	14.1±1.44^{ab}	14.5±1.17^{ab}	15.5±1.53^{b}	0.01	NS	NS
18:2ω6	59.1±4.99^{a}	56.0±5.41^{a}	57.4±4.70^{a}	49.8±4.96^{b}	0.009	0.001	NS
20:4ω6	2.4±0.64	2.1±0.47	2.3±0.81	2.2±0.33	NS	NS	NS
Total SFA[3]	9.7±2.69	10.4±3.69	9.8±1.81	12.2±4.12	NS	NS	NS
Total MUFA[3]	24.9±2.68^{a}	25.9±2.83^{a}	26.2±2.66^{a}	30.8±3.48^{b}	0.001	0.002	0.04
Total PUFA[3]	69.0±4.80^{a}	66.4±2.68^{a}	66.6±3.10^{a}	58.9±4.87^{b}	0.0001	0.0001	0.04
18:2ω6+ 20:4ω6	61.4±4.82^{a}	58.1±5.53^{a}	59.7±4.14^{a}	52.0±5.01^{b}	0.008	0.0001	NS
P/S	7.5±1.85^{a}	7.0±2.02^{a}	7.0±1.40^{a}	5.3±1.61^{b}	0.04	0.03	NS

[1]Values are mean ± standard deviation of 12 individual animals for each dietary treatment.
[2]Values, within rows, without common superscripts are significantly different according to Student-Newman-Keuls' Multiple Range Test, ($p<0.05$).
[3]Abbreviations used: P/S = polyunsaturated to saturated fatty acid ratio; NS = no significance; SFA = total saturated fatty acids; MUFA = total monounsaturated fatty acids; PUFA = total poyunsaturated fatty acids.
[4]Int., interaction between effects of dietary fat level and polyunsaturated to saturated fatty acid ratio.

20:4ω6 levels and polyunsaturated to saturated fatty acid ratio of cardiolipin fatty acids increased by 8% ($p<0.001$), 9% ($p<0.001$) and 14% ($p<0.03$), respectively, in rats fed diets with polyunsaturated to saturated fatty acid ratios of 1.35. Polyunsaturated to saturated fatty acid ratio of diets had no effect on 16:0, 18:0, 18:1ω7, 20:4ω6 and total saturated fatty acid content of cardiolipin.

(d) Phosphatidylinositol

18:0, comprising greater than 43% of total phosphatidylinositol fatty acids, and 20:4 ω6, comprising greater than 36% of total phosphatidylinositol fatty acids, were the major fatty acids found comprising phosphatidylinositol (table 8). Diets high in fat increased mitochondrial membrane phosphatidylinositol levels of 18:0 by 7% ($p<0.04$), 20:4 ω6 by 13% ($p<0.001$), total polyunsaturated ω3 and ω6 fatty acids by 6% ($p<0.01$), and the sum of 18:2ω6 and 20:4ω6 levels by 13% ($p<0.001$). High fat diets lowered phosphatidylinositol levels of 16:0 by 22% ($p<0.01$), and total monounsaturated fatty acids by 23% ($p<0.006$). 18:1, 18:2ω6, total saturated fatty acid levels and polyunsaturated to saturated fatty acid ratio of phosphatidylinositol were not altered by dietary fat levels. Polyunsaturated to saturated fatty acid ratio of diets had no effect on fatty acid composition of phosphatidylinositol, except dietary polyunsaturated to saturated fatty acid ratios of 1.35 lowered phosphatidylinositol total monounsaturated fatty acid levels by 17% ($p<0.04$).

Table 8. Effect of High versus Low Fat Diets and High versus Low Dietary Polyunsaturated to Saturated Fatty Acid Ratios on Liver Mitochondrial Membrane Phosphatidylinositol Fatty Acid Composition

Fatty Acid	Fatty Acid Composition, (%w/w)[1,2]				Two-way Analysis of Variance (p<)		
	High Fat Diet		Low Fat Diet				
	P/S[3]=1.35	P/S=0.35	P/S=1.35	P/S=0.35	Fat	P/S	Int.[4]
16:0	4.7±0.91[a]	5.3±1.78[ab]	6.2±1.72[ab]	6.7±2.34[b]	0.01	NS[3]	NS
18:0	45.4±2.85	45.2±2.14	42.4±4.19	42.2±7.59	0.04	NS	NS
18:1ω9	1.1±0.23	1.3±0.34	1.3±0.45	1.5±0.59	NS	NS	NS
18:1ω7	1.2±0.15	1.3±0.19	1.2±0.19	1.2±0.45	NS	NS	NS
18:2ω6	2.4±0.69	2.3±0.95	2.2±0.66	1.6±0.72	NS	NS	NS
20:4ω6	39.5±1.55[ab]	39.4±2.31[a]	35.5±6.48[bc]	33.2±4.05[c]	NS	NS	NS
Total SFA[3]	51.0±1.93	50.9±1.68	50.2±2.55	52.4±5.96	NS	NS	NS
Total MUFA[3]	3.4±0.77[a]	3.9±0.58[a]	4.2±1.30[a]	5.3±1.99[b]	0.006	0.04	NS
Total PUFA[3]	46.8±1.51	46.4±1.49	43.8±5.65	43.8±4.37	0.01	NS	NS
18:2ω6+ 20:4ω6	41.9±1.64[a]	41.7±2.14[a]	37.8±6.08[b]	34.8±3.94[b]	0.0001	NS	NS
P/S	0.9±0.06	0.9±0.06	0.9±0.13	0.9±0.15	NS	NS	NS

[1] Values are mean ± standard deviation of 12 individual animals for each dietary treatment.

[2] Values, within rows, without common superscripts are significantly different according to Student-Newman-Keuls' Multiple Range Test, ($p<0.05$).

[3] Abbreviations used: P/S = polyunsaturated to saturated fatty acid ratio; NS = no significance; SFA = total saturated fatty acids; MUFA = total monounsaturated fatty acids; PUFA = total polyunsaturated fatty acids.

[4] Int., interaction between effects of dietary fat level and polyunsaturated to saturated fatty acid ratio.

(e) Sphingomyelin

Sphingomyelin contained 18:2ω6, 20:4ω6 and saturated fatty acids representing greater than 8%, 12%, and 56% (w/w), respectively, of total fatty acids in sphingomyelin (table 9). Diets providing 40% of calories as fat decreased sphingomyelin content of 18:1ω9 by 2% ($p<0.01$) and 18:1ω7 by 24% ($p<0.001$). Dietary fat levels had no effect on 16:0, 18:0, 18:2ω6, 20:4ω6, total saturated fatty acid, total monounsaturated fatty acid, total polyunsaturated ω3 and ω6 fatty acid, the sum of 18:2ω6 and 20:4ω6 levels and polyunsaturated to saturated fatty acid ratios of mitochondrial membrane sphingomyelin.

Diets with polyunsaturated to saturated fatty acid ratios of 1.35 increased sphingomyelin levels of 18:2ω6, 20:4ω6 and the sum of 18:2ω6 and 20:4ω6 levels by 18% ($p<0.05$), 19% ($p<0.02$) and 19% ($p<0.001$), respectively. Sphingomyelin levels of 18:1ω9 and total monounsaturated fatty acids were reduced by 28% ($p<0.001$) and 19% ($p<0.004$), respectively, in rats fed diets with a polyunsaturated to saturated fatty acid ratio of 1.35. Dietary polyunsaturated to saturated fatty acid ratio had no effect on levels of 16:0, 18:0, 18:1ω7, total saturated fatty acid, total polyunsaturated ω3 and ω6 fatty acid and polyunsaturated to saturated fatty acid ratio of sphingomyelin.

(f) Phosphatidylserine

The main fatty acids of phosphatidylserine were found to be 20:4ω6, comprising greater than 20% (w/w), and saturated fatty acids, comprising greater than 52% (w/w) of mitochondrial membrane phosphatidylserine fatty acids (table 10). Dietary fat level affected

Table 9. Effect of High versus Low Fat Diets and High versus Low Dietary Polyunsaturated to Saturated Fatty Acid Ratio on Liver Mitochondrial Membrane Sphingomyelin Fatty Acid Composition

Fatty Acid	Fatty Acid Composition, (%w/w)[1,2]				Two-way Analysis of Variance (p<)		
	High Fat Diet		Low Fat Diet				
	P/S[3]=1.35	P/S=0.35	P/S=1.35	P/S=0.35	Fat	P/S	Int.[4]
16:0	27.3±5.36	26.7±5.08	28.4±3.24	29.0±3.90	NS[3]	NS	NS
18:0	24.9±3.50	24.5±4.93	23.6±2.26	22.3±4.04	NS	NS	NS
18:1ω9	5.0±0.97[a]	5.9±2.16[a]	5.3±0.91[a]	8.3±2.51[b]	0.01	0.0001	0.04
18:1ω7	2.1±0.50[a]	2.1±0.64[a]	2.5±0.60[ab]	2.9±0.75[b]	0.001	NS	NS
18:2ω6	9.7±3.00	7.5±2.60	9.1±3.38	8.0±2.97	NS	0.05	NS
20:4ω6	14.2±4.81[a]	12.9±2.75[ab]	14.7±2.87[a]	10,5±3.87[bc]	NS	0.02	NS
Total SFA[3]	57.3±6.63	57.8±6.17	56.4±2.11	55.2±3.89	NS	NS	NS
Total MUFA[3]	12.4±3.40[a]	15.1±3.53[ab]	12.9±2.46[a]	16.4±4.19[bc]	NS	0.004	NS
Total PUFA[3]	30.6±8.33	27.9±4.45	31.0±2.13	26.4±7.94	NS	NS	NS
18:2ω6+ 20:4ω6	23.9±6.51[a]	20.4±3.61[ab]	23.8±3.43[a]	18.4±3.90[bc]	NS	0.001	NS
P/S	0.6±0.20	0.5±0.12	0.6±0.05	0.5±0.18	NS	NS	NS

[1] Values are mean ± standard deviation of 12 individual animals for each dietary treatment.
[2] Values, within rows, without common superscripts are significantly different according to Student-Newman-Keuls' Multiple Range Test, ($p<0.05$).
[3] Abbreviations used: P/S = polyunsaturated to saturated fatty acid ratio; NS = no significance; SFA = total saturated fatty acids; MUFA = total monounsaturated fatty acids; PUFA = total polyunsaturated fatty acids.
[4] Int., interaction between effects of dietary fat level and polyunsaturated to saturated fatty acid ratio.

Table 10. Effect of High versus Low Fat Diets and High versus Low Dietary Polyunsaturated to Saturated Fatty Acid Ratio on Liver Mitochondrial Membrane Phosphatidylserine Fatty Acid Composition

Fatty Acid	Fatty Acid Composition, (%w/w)[1,2]				Two-way Analysis of Variance (p<)		
	High Fat Diet		Low Fat Diet				
	P/S[3]=1.35	P/S=0.35	P/S=1.35	P/S=0.35	Fat	P/S	Int.[4]
16:0	14.7±4.05	15.7±5.81	17.0±3.36	16.4±5.21	NS[3]	NS	NS
18:0	35.9±3.46	33.2±4.97	34.8±4.01	30.8±5.85	NS	NS	NS
18:1ω9	4.0±0.52^{a}	5.4±1.71^{b}	4.3±0.50^{ab}	4.4±0.66^{a}	0.05	NS	NS
18:1ω7	2.0±0.29^{a}	2.4±0.52^{ab}	2.7±0.47^{bc}	3.1±0.59^{c}	0.0001	0.02	NS
18:2ω6	4.6±1.34	4.4±1.30	4.7±1.14	3.8±1.31	NS	NS	NS
20:4ω6	21.6±2.36^{ab}	19.7±4.96^{ab}	23.0±2.36^{a}	18.0±4.31^{b}	NS	0.01	NS
22:6ω3	6.1±1.37	5.8±1.89	6.8±1.22	5.2±1.36	NS	NS	NS
Total SFA[3]	53.2±4.34	52.2±6.37	54.0±3.89	52.4±5.74	NS	NS	NS
Total MUFA[3]	11.2±1.80	13.3±3.47	10.7±2.94	14.1±2.25	NS	0.004	NS
Total PUFA[3]	34.9±4.02	32.4±3.74	36.6±4.89	33.5±3.12	NS	0.03	NS
18:2ω6+ 20:4ω6	26.2±3.16^{ab}	24.1±2.92^{ac}	27.7±3.75^{b}	21.8±1.70^{c}	NS	0.0001	NS
P/S	0.7±0.12	0.6±0.18	0.7±0.10	0.7±0.13	NS	NS	NS

[1] Values are mean ± standard deviation of 12 individual animals for each dietary treatment.
[2] Values, within rows, without common superscripts are significantly different according to Student-Newman-Keuls' Multiple Range Test, ($p<0.05$).
[3] Abbreviations used: P/S = polyunsaturated to saturated fatty acid ratio; NS = no significance; SFA = total saturated fatty acids; MUFA = total monounsaturated fatty acids; PUFA = total polyunsaturated fatty acids.
[4] Int., interaction between effects of dietary fat level and polyunsaturated to saturated fatty acid ratio.

only 18:1ω9 and 18:1ω7 content of phosphatidylserine. High fat diets lowered phosphatidylserine 18:1ω7 levels by 25% ($p<0.001$) and increased 18:1ω9 levels by 7% ($p<0.05$).

Diets with high polyunsaturated to saturated fatty acid ratios increased phosphatidylserine levels of 20:4ω6 by 15% ($p<0.01$), total polyunsaturated ω3 and ω6 fatty acids by 8% ($p<0.03$), and the sum of 18:2ω6 and 20:4ω6 levels by 15% ($p<0.01$). Phosphatidylserine levels of 18:1ω7 and total monounsaturated fatty acies were lowered by 14% ($p<0.02$) and 20% ($p<0.004$), respectively, by feeding diets with polyunsaturated to saturated fatty acid ratios of 1.35. Polyunsaturated to saturated fatty acid ratio of dietary fat had no effect on 16:0, 18:0, 18:2ω6 and 22:6ω3 levels of phosphatidylserine.

(g) Phosphatidylglycerol

The major fatty acids found comprising phosphatidyl-glycerol were 18:1, 18:2ω6, 20:4ω6, and saturated fatty acids (table 11). The above fatty acids comprised greater than 14%, 35%, 10%, and 27% (w/w), respectively, of total phosphatidylglycerol fatty acids. Diets providing 40% of calories as fat increased 18:2 ω6, total polyunsaturated ω3 and ω6 fatty acid and the sum of 18:2ω6 and 20:4ω6 levels of mitochondrial membrane phosphatidylglycerol by 14% ($p<0.005$), 7% ($p<0.007$) and 10% ($p<0.004$), respectively. Phosphatidylglycerol levels of total monounsaturated fatty acid decreased by 10% ($p<0.05$) in rats fed high fat diets. 18:1ω7 levels were lowered by 3% ($p<0.01$) and the sum of 18:2ω6 and 20:4ω6 levels were increased by 7% ($p<0.04$) in phosphatidylglycerol by diets providing a polyunsaturated to saturated

Table 11. Effect of High versus Low Fat Diets and High versus Low Dietary Polyunsaturated to Saturated Fatty Acid Raio on Liver Mitochondrial Membrane Phosphatidylglycerol Fatty Acid Composition

Fatty Acid	Fatty Acid Composition, (%w/w)[1,2]				Two-way Analysis of Variance (p<)		
	High Fat Diet		Low Fat Diet				
	P/S[3]=1.35	P/S=0.35	P/S=1.35	P/S=0.35	Fat	P/S	Int.[4]
16:0	9.0±2.90	9.7±3.07	10.5±5.94	9.9±3.06	NS[3]	NS	NS
18:0	14.6±3.14	14.5±3.00	14.4±3.87	14.2±3.02	NS	NS	NS
18:1ω9	5.8±1.75^{ab}	6.1±1.20^{ab}	5.3±0.90^{a}	7.1±1.23^{b}	NS	0.01	NS
18:1ω7	8.0±1.55	8.1±1.27	8.3±1.80	8.7±1.68	NS	NS	NS
18:2ω6	38.9±5.54^{a}	37.0±6.72^{a}	35.0±7.77^{ab}	30.3±4.75^{bc}	0.005	NS	NS
20:4ω6	9.6±1.85	10.0±2.26	10.4±3.57	10.1±2.66	NS	NS	NS
Total SFA[3]	26.4±4.49	27.3±5.47	27.6±7.71	29.5±4.67	NS	NS	NS
Total MUFA[3]	17.3±3.17^{a}	16.7±2.85^{a}	17.3±3.52^{a}	20.5±3.37^{b}	0.05	NS	0.05
Total PUFA[3]	58.0±5.60^{ab}	57.8±5.04^{a}	55.0±5.49^{ab}	52.4±3.58^{b}	0.007	NS	NS
18:2ω6+ 20:4ω6	48.5±5.42^{a}	47.0±5.87^{a}	45.4±6.33^{a}	30.4±4.05	0.004	0.04	NS
P/S	2.3±0.46	2.3±0.84	2.2±0.77	1.8±0.37	NS	NS	NS

[1] Values are mean ± standard deviation of 12 individual animals for each dietary treatment.
[2] Values, within rows, without common superscripts are significantly different according to Student-Neuman-Keuls' Multiple Range Test, ($p<0.05$).
[3] Abbreviations used: P/S = polyunsaturated to saturated fatty acid ratio; NS = no significance; SFA = total saturated fatty acids; MUFA = total monounsaturated fatty acids; PUFA = total polyunsaturated fatty acids.
[4] Int., interaction between effects of dietary fat level and polyunsaturated to saturated fatty acid ratio.

fatty acid ratios of 1.35. No other effects of dietary fatty acid composition on fatty acid composition of phosphatidylglycerol were noted.

(h) Total Phospholipid

Hepatic mitochondrial membrane total phospholipids contained 18:0, 18:2 ω6 and 20:4ω 6 representing greater than 25%, 19%, and 21%, respectively, of total fatty acids in total phospholipids (table 12). Diets providing 40% of calories as fat increased total phospholipid levels of 18:0 by 8% ($p<0.006$), 18:2ω6 by 10% ($p<0.001$), 20:4 ω6 by 7% ($p<0.004$), total polyunsaturated ω3 and ω6 fatty acid by 5% ($p<0.001$) and the sum of 18:2ω6 and 20:4 ω6 levels by 9% ($p<0.003$). High fat diets decreased total phospholipid levels of 16:0 by 18% ($p<0.001$), 18:1ω9 by 11% ($p<0.001$), 18:1 ω7 by 8% ($p<0.006$), total saturated fatty acid by 2% ($p<0.001$) and total monounsaturated fatty acid by 11% ($p<0.003$). Dietary fat levels had no significant effects on total phospholipid polyunsaturated to saturated fatty acid ratios.

Dietary polyunsaturated to saturated fatty acid ratio had no effect on total phospholipid levels of 16:0, 18:0, 18:1 ω7 and 20:4ω 6. Diets with poyunsaturated to saturated fatty acid ratio of 1.35 increased total phospholipid levels of 18:2ω6 by 12% ($p<0.001$), total polyunsaturated ω3 and ω6 fatty acid by 4% ($p<0.001$) and the sum of 18:2 ω6 and 20:4 ω6 levels by 7% ($p<0.003$). Levels of 18:1 ω9, total saturated fatty acid and total monounsaturated fatty acid were decreased by 16% ($p<0.001$), 3% ($p<0.001$) and 11% ($p<0.001$), respectively, in rats fed diets providing polyunsaturated to saturated

Table 12. Effect of High versus Low Fat Diets and High versus Low Dietary Polyunsaturated to Saturated Fatty Acid Ratio on Liver Mitochondrial Membrane Total Phospholipid Fatty Acid Composition

Fatty Acid	Fatty Acid Composition, (%w/w)[1,2]				Two-way Analysis of Variance (p<)		
	High Fat Diet		Low Fat Diet				
	P/S[3]=1.35	P/S=0.35	P/S=1.35	P/S=0.35	Fat	P/S	Int.[4]
16:0	12.0±2.00[a]	12.5±1.20[a]	14.7±1.30[b]	15.2±1.50[b]	0.0001	NS[3]	NS
18:0	25.1±2.70	26.0±2.20	23.4±2.20	23.6±2.80	0.006	NS	NS
18:1ω9	4.1±0.50[a]	4.7±0.60[b]	4.3±0.40[a]	5.6±0.50[c]	0.001	0.0001	0.01
18:1ω7	4.6±0.70[a]	4.5±0.50[a]	4.8±0.50[ab]	5.1±0.50[b]	0.006	NS	NS
18:2ω6	19.4±1.80[a]	17.3±1.90[b]	17.6±1.80[b]	15.4±2.00[c]	0.001	0.0001	NS
20:4ω6	21.4±1.00[a]	21.4±2.07[a]	20.5±1.80[ab]	19.2±1.90[b]	0.004	NS	NS
Total SFA[3]	38.5±4.20[a]	40.0±2.90[a]	39.5±2.60[a]	40.8±3.50[b]	0.0001	0.0001	0.006
Total MUFA[3]	11.0±1.05[a]	11.5±1.10[b]	11.5±1.10[b]	13.9±1.40[c]	0.0001	0.001	NS
Total PUFA[3]	50.6±2.20[a]	48.8±2.50[b]	48.2±1.30[b]	45.7±1.90[c]	0.0001	0.0001	NS
18:2ω6+ 20:4ω6	40.8±1.80[a]	38.7±1.70[ab]	38.0±1.70[bc]	34.5±2.00[c]	0.003	0.003	NS
P/S	1.3±0.14[a]	1.2±0.09[ab]	1.2±0.09[bc]	1.1±0.10[c]	NS	NS	NS

[1] Values are mean ± standard deviation of 12 individual animals for each dietary treatment.
[2] Values, within rows, without common superscripts are significantly different according to Student-Newman-Keuls' Multiple Range Test, ($p<0.05$).
[3] Abbreviations used: P/S = polyunsaturated to saturated fatty acid ratio; NS = no significance; SFA = total saturated fatty acids; MUFA = total monounsaturated fatty acids; PUFA = total polyunsaturated fatty acids.
[4] Int., interaction between effects of dietary fat level and polyunsaturated to saturated fatty acid ratio.

fatty acid ratio of 1.35.

III. EFFECT OF DIET ON HEPATIC CARNITINE PALMITOYLCARNITINE TRANSLOCASE AND CARNITINE PALMITOYLTRANSFERASE-1 FUNCTIONS

Carnitine palmitoylcarnitine translocase and carnitine palmitoyltransferase-1 activities were each assessed at six different substrate concentration levels. Activities observed at these six concentration levels were used to calculate carnitine palmitoylcarnitine translocase velocity (table 13) and Km for carnitine palmitoyltransferase-1 (tables 14).

(a) Carnitine Palmitoylcarnitine Translocase

Although carnitine palmitoylcarnitine translocase activity, assessed over all substrate concentrations by repeated measures analysis of variance, increased by 28% in rats fed high fat diets the increase in activity was not significant (table 13). Carnitine palmitoylcarnitine translocase activity at substrate concentration of 4 nmol palmitoylcarnitine was 19% ($p<0.03$) higher in rats fed high fat diets than in rats fed low fat diets. Although, carnitine palmitoylcarnitine translocase velocity determined by activity versus substrate concentration plots was 6% higher in rats fed high fat diets than in rats fed low fat diets, velocity was not significantly altered by dietary fat level. Carnitine palmitoylcarnitine translocase velocity, activity at substrate concentration of 4 nmol and activity determined by repeated measures analysis of variance were not affected

Table 13. Effect of High versus Low Fat Diets and High versus Low Dietary Polyunsaturated to Saturated Fatty Acid Ratios on Hepatic Carnitine Palmitoylcarnitine Translocase Functions

Function	Function Measurements[1,2]				Analysis of Variance (p<)		
	High Fat Diet		Low Fat Diet				
	P/S[3]=1.35	P/S=0.35	P/S=1.35	P/S=0.35	Fat	P/S	Int.[4]
Total Activity[5]	316 ±777	249 ±172	194 ±167	214 ±148	NS[3]	NS	NS
Activity at 4nmol substrate[6]	189 ± 65.5	204 ± 76.5	149 ± 60.5	171 ± 55.5	0.03	NS	NS
Velocity[7]	40.8± 15.7	47.4± 18.7	40.6 ± 24.0	42.0± 16.0	NS	NS	NS

[1] Values are mean ± standard deviation.
[2] No two groups were significantly different according to Student-Newman-Keuls' Multiple Range Test (p<0.05).
[3] Abbreviations used: P/S = polyunsaturated to saturated fatty acid ratio; NS = no significance.
[4] Int., interaction between effects of dietary fat level and polyunsaturated to saturated fatty acid ratio.
[5] Activity, (pmol/min./mg prot.), observed at six concentration levels of palmitoylcarnitine substrate were compared using repeated measures analysis of variance, n=72.
[6] Activity, (pmol/min./mg prot.), observed at concentration level of 4 nmol palmitoylcarnitine substrate were compared using twoway analysis of variance, n=12.
[7] Velocity, (pmol/min./mg prot./nmol substrate), was determined by activity versus palmitoylcarnitine substrate concentration plots, n=12.

Table 14. Effect of High versus Low Fat Diets and High versus Low Dietary Polyunsaturated to Saturated Fatt Acid Ratios on Hepatic Carnitine Palmitoyltransferase-1 Functions

Function	Function Measurements[1,2]				Analysis of Variance (p<)		
	High Fat Diet		Low Fat Diet				
	P/S[3]=1.35	P/S=0.35	P/S=1.35	P/S=0.35	Fat	P/S	Int.[4]
Total Activity[5]	983±484	1078±460	973 ±446	1041 ±373	0.04	NS[3]	NS
Activity at 40 nmol Substrate[6]	525±121	579±139	523 ±153	641 ±123	NS	0.03	NS
Km[7]	103± 43.0	111± 61.0	97.8± 36.4	68.0± 34.1	NS	NS	NS

[1] Values are mean ± standard deviation.
[2] No two groups were significantly different according to Student-Newman-Keuls' Multiple Range Test, (p<0.05).
[3] Abbreviations used: P/S = polyunsaturated to saturated fatty acid ratio; NS = no significance.
[4] Int., interaction between effects of dietary fat level and polyunsaturated to saturated fatty acid ratios.
[5] Activity, (pmol/min./mg prot.), observed at six concentration levels of carnitine substrate were compared using repeated measures of analysis of variance, n=72.
[6] Activity, (pmol/min./mg prot.), observed at concentration level 40 nmol carnitine substrate concentration were compared using two-way analysis of variance, n=12.
[7] Km, (uM), was determined by Eadie-Hofstee plots, n=12.

by dietary polyunsaturated to saturated fatty acid ratio.

(b) Carnitine Palmitoyltransferase-1

Repeated measures analysis of variance assessed over all substrate concentrations showed that carnitine palmitoyltransferase-1 activity increased by 2% ($p<0.04$) in rats fed high fat diets (table 14). Dietary fat levels did not significantly alter carnitine palmitoyltransferase-1 activity at 40 nmol substrate concentration. Although, carnitine palmitoyltransferase-1 Km determined by Eadie-Hofstee plots was 22% higher in rats fed high fat diets than in rats fed low fat diets, Km was not significantly altered by dietary fat levels. Carnitine palmitoyltransferase-1 activity analyzed by repeated measures of analysis of variance, although not significantly different, was 7% higher in rats fed diets with polyunsaturated to saturated fatty acid ratios of 0.35. Carnitine palmitoyltransferase-1 activity at substrate concentration of 40 nmol was 14% ($p<0.03$) higher in rats fed diets with polyunsaturated to saturated fatty acid ratio of 0.35 than in rats fed diets with polyunsaturated to saturated fatty acid ratio of 1.35. Dietary polyunsaturated to saturated fatty acid ratio had no significant effect on carnitine palmitoyltransferase-1 Km values.

IV. RELATIONSHIP BETWEEN MITOCHONDRIAL MEMBRANE PHOSPHOLIPID DISTRIBUTION ON CARNITINE PALMITOYLCARNITINE TRANSLOCASE AND CARNITINE PALMITOYLTRANSFERASE-1 FUNCTIONS

To examine the relationship between membrane phospholipid compositional changes and membrane protein activity, scatterplots and

regression lines were constructed to illustrate mitochondrial carnitine palmitoylcarnitine translocase functions versus mitochondrial membrane phospholipid levels and carnitine palmitoyltransferase-1 functions versus mitochondrial membrane phospholipid levels. Regression equations, correlation coefficients (r), and significance of membrane phospholipid level correlation coefficients (p) with carnitine palmitoylcarnitine translocase functions are presented (table 15). Correlations of membrane phospholipid levels versus carnitine palmitoyltransferase-1 functions are also presented (Table 16).

(a) Carnitine Palmitoylcarnitine Translocase

Increased carnitine palmitoylcarnitine translocase activity at substrate concentration of 4 nmol ($r=0.51$, $p<0.00009$) and carnitine palmitoylcarnitine translocase velocity ($r=0.60$, $p<0.00001$, figure 1) corresponded with increasing cardiolipin levels. Carnitine palmitoylcarnitine translocase velocity and activity at 4 nmol substrate concentration also correlated with increasing phosphatidylserine (velocity: $r=0.48$, $p<0.0003$, activity: $r=0.35$, $p<0.007$), phosphatidylinositol (velocity: $r=0.30$, $p<0.02$, activity: $r=0.32$, $p<0.01$) and phosphatidylglycerol (velocity: $r=0.39$, $p<0.003$, activity: $r=0.32$, $p<0.01$) levels (results not shown). It logically followed that carnitine palmitoylcarnitine translocase velocity ($r=0.59$, $p<0.00001$) and activity at substrate concentration of 4 nmol ($r=0.52$, $p<0.00009$) increased as total acidic phospholipid levels increased. Carnitine palmitoylcarnitine translocase velocity and activity at 4 nmol substrate concentration also increased as

Table 15. Regression Equations Representing the Relationship Between Liver Mitochondrial Membrane Phospholipid Levels and Carnitine Palmitoylcarnitine Translocase Function

Function	Phospholipid (ug/mg protein)	Linear Regression Equation	r[1]	p<[2]
Activity at 4 nmol Substrate[3]	Cardiolipin	y= 3.20x+ 28.6	0.51	0.00009
	Acidic phospholipids	y= 1.38x+ 33.8	0.52	0.00009
	Neutral phospholipids	y= 0.98x+ 50.8	0.38	0.004
	Neutral to acidic phospholipid ratio	y=-81.2 x-283	-0.27	0.03
	Membrane total phospholipids	y= 0.65x+ 26.1	0.45	0.0006
Velocity[4]	Cardiolipin	y= 1.01x- 4.93	0.60	0.000001
	Acidic phospholipids	y= 0.43x- 2.79	0.59	0.000001
	Neutral phospholipids	y= 0.33x- 0.90	0.47	0.0004
	Neutral to acidic phospholipid ratio	y=-23.3 x- 72.5	-0.28	0.03
	Membrane total phospholipids	y= 0.22x- 9.88	0.57	0.00001

[1] [2] r, regression correlation coefficient.
[3] p<, significance of correlation.
Values are given for linear regression over all diet treatment groups between y, carnitine palmitoylcarnitine translocase activity at 4 nmol substrate (pmol/min./mg prot.) versus x, liver mitochondrial
[4] membrane phospholipid levels (ug/mg prot.), n=48.
Values are given for linear regression over all diet treatment groups between y, carnitine palmitoylcarnitine translocase velocity (pmol/min./mg prot./nmol palmitoylcarnitine) determined from activiy versus palmitoylcarnitine substrate concentration plots versus x, liver mitochondrial membrane phospholipid levels (ug/mg prot.), n=48.

Table 16. Regression Equations Representing the Relationship Between Liver Mitochondrial Membrane Phospholipid Levels and Carnitine Palmitoyltransferase-1 Function

Function	Phospholipid (ug/mg protein)	Linear Regression Equation	r[1]	p<[2]
Activity at 40 nmol Substrate[3]	Cardiolipin	y= 3.66x+402	0.27	0.03
	Phosphatidylcholine	y= 2.59x+433	0.28	0.03
	Phosphatidyl-ethanolamine	y= 3.60x+365	0.30	0.02
	Neutral phospholipids	y= 2.02x+340	0.33	0.01
Km[4]	Cardiolipin	y= 5.43x+ 71.3	0.24	0.05
	Neutral to Acidic (without cardiolipin) phospholipid ratio	y=32.2 x+ 18.2	0.35	0.007

[1] r, regression correlation coefficient.
[2] p<, significance of correlation.
[3] Values are given for linear regression over all diet treatment groups between y, carnitine palmitoyltransferase-1 activity at 40 nmol substrate (pmol/min./mg prot.) versus x, liver mitochondrial membrane phospholipid levels, (ug/ mg prot.),n=48.
[4] Values are given for linear regression over all diet treatment groups between y, carnitine palmitoyltransferase-1 Km (uM) determined from Eadie-Hofstee plots versus x, liver mitochondrial membrane phospholipid levels (ug/mg prot.), n=48.

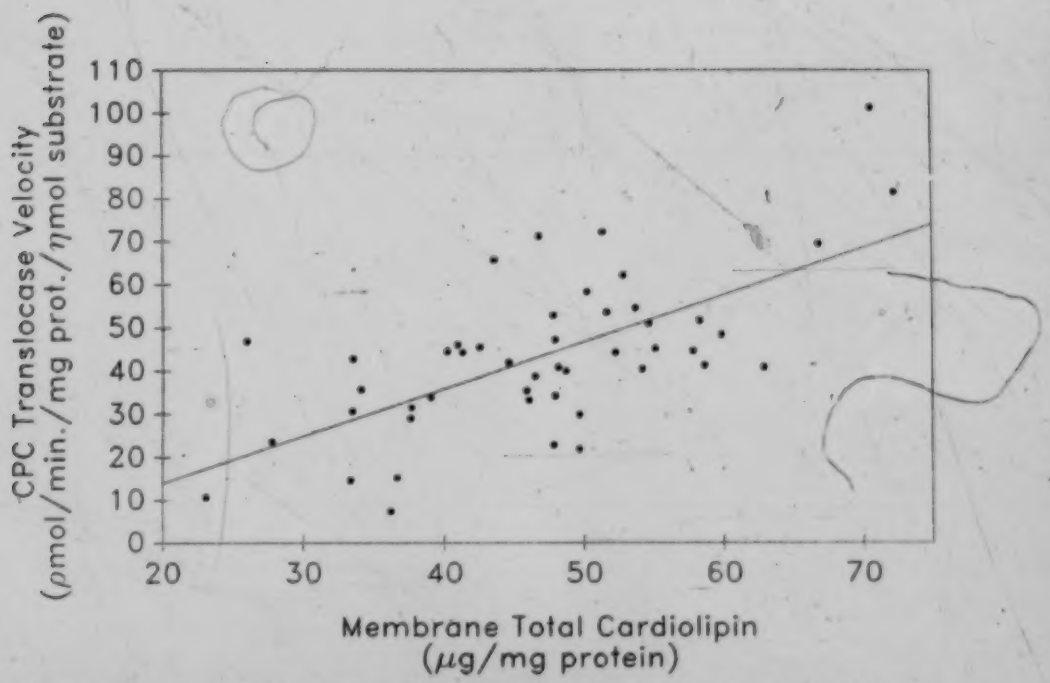

Figure 1. Regression Line Representing the Relationship Between Hepatic Mitochondrial Membrane Cardiolipin Levels and Carnitine Palmitoylcarnitine Translocase Velocity. Carnitine palmitoylcarnitine translocase velocities were determined from activity versus substrate concentration plots for individual animals. Data from individual animals from each dietary treatment group were used to construct the regression line $y = 1.01x - 4.93$ ($r = 0.60$, $p < 0.00001$, $n = 48$).

phosphatidylcholine (velocity: $r=0.33$, $p<0.01$, activity: $r=0.26$, $p<0.04$) and phosphatidylethanolamine (velocity: $r=0.53$, $p<0.00005$, activity: $r=0.47$, $p<0.0004$) levels increased (results not shown). It logically followed that carnitine palmitoylcarnitine translocase velocity ($r=0.47$, $p<0.0004$, figure 2) and activity at 4 nmol substrate concentration ($r=0.38$, $p<0.004$) increased as mitochondrial membrane total neutral phospholipid levels increased. Increasing carnitine palmitoylcarnitine translocase velocity ($r=-0.28$, $p<0.03$) and activity at 4 nmol substrate concentration ($r=-0.27$, $p<0.03$) correlated with decreasing total neutral to total acidic phospholipid ratios. Carnitine palmitoylcarnitine translocase velocity ($r=0.57$, $p<0.00001$) and activity at 4 nmol substrate concentration ($r=0.45$, $p<0.0006$) increased with increasing total phospholipid content of membranes.

(b) Carnitine Palmitoyltransferase-1

Carnitine palmitoyltransferase-1 activity at 40 nmol substrate concentration ($r=0.27$, $p<0.03$) and carnitine palmitoyltransferase-1 Km ($r=0.24$, $p<0.05$) increased as cardiolipin levels increased. Total acidic phospholipid levels were not significantly correlated with either carnitine palmitoyltransferase-1 activity at 40 nmol substrate concentration or carnitine palmitoyltransferase-1 Km. But, carnitine palmitoyltransferase-1 Km decreased as the sum of phosphatidylinositol, phosphatidylserine and phosphatidylglycerol levels increased ($r=-0.24$, $p<0.05$). Carnitine palmitoyltransferase-1 activity at 40 nmol substrate concentration level increased as levels of phosphatidylcholine ($r=0.28$, $p<0.03$), phosphatidylethanolamine ($r=0.30$, $p<0.02$), and total neutral phospholipid ($r=0.33$, $p<0.01$) increased. In

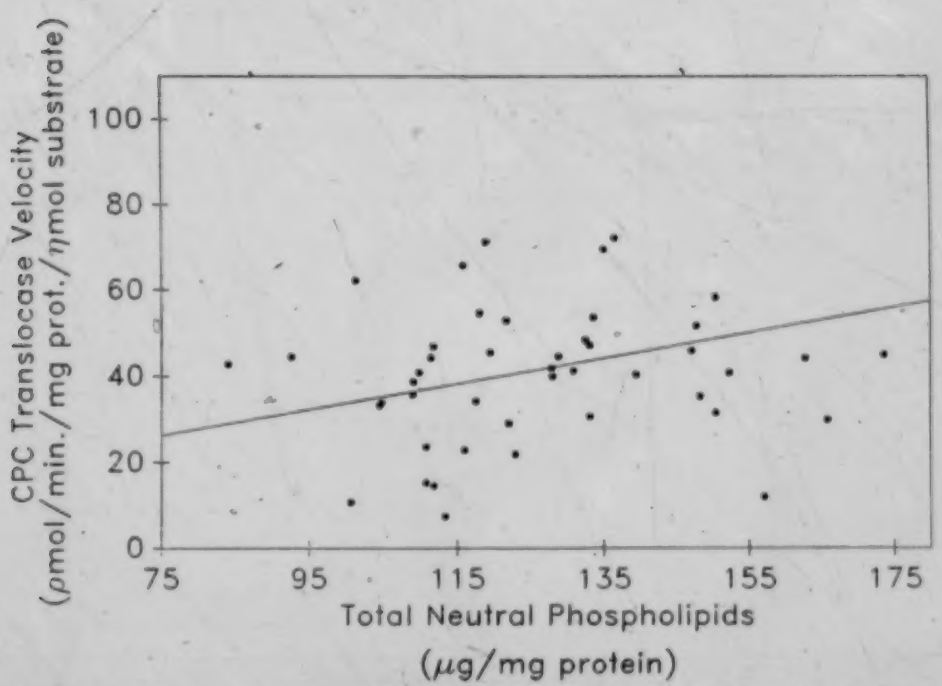

Figure 2. Regression Line Representing the Relationship Between Hepatic Mitochondrial Membrane Total Neutral Phospholipid Levels and Carnitine Palmitoylcarnitine Translocase Velocity. Carnitine palmitoylcarnitine translocase velocities were determined from activity versus substrate concentration plots for individual animals. Data from individual animals from each dietary treatment group were used to construct the regression line $y=0.33x-0.90$ ($r=0.47$, $p<0.0004$, $n=48$).

the present study, carnitine palmitoyltransferase-1 Km increased as total neutral phospholipids versus the sum of phosphatidylinositol, phosphatidylserine and phosphatidylglycerol levels ratio increased ($r=0.35$, $p<0.007$).

V. RELATIONSHIP BETWEEN LIVER MITOCHONDRIAL MEMBRANE PHOSPHOLIPID FATTY ACID COMPOSITION AND CARNITINE PALMITOYLCARNITINE TRANSLOCASE AND CARNITINE PALMITOYLTRANSFERASE-1 FUNCTIONS

To examine the relationship between membrane phospholipid fatty acid compositional changes and activity of membrane protein, scatterplots and regression lines were constructed to illustrate mitochondrial carnitine palmitoylcarnitine translocase functions versus mitochondrial membrane phospholipid fatty acid levels and carnitine palmitoyltransferase-1 functions versus mitochondrial membrane phospholipid fatty acid levels. Regression equations, correlation coefficients (r), and significance of phospholipid fatty acid level correlation coefficients (p) with carnitine palmitoylcarnitine translocase functions are presented (table 17). Correlations of phospholipid fatty acid levels versus carnitine palmitoyltransferase-1 functions are also presented (table 18).

(a) Carnitine Palmitoylcarnitine Translocase

Increasing levels of 18:0 from phosphatidylcholine ($r=0.41$, $p<0.002$, figure 3), phosphatidylethanolamine ($r=0.34$, $p<0.009$, results not shown) and total phospholipids ($r=0.34$, $p<0.008$) correlated with

Table 17. Regression Equations Representing the Relationship Between Liver Mitochondrial Membrane Phospholipid Fatty Acid Levels and Carnitine Palmitoylcarnitine Translocase Function

Function	Fatty Acid (% phospholipid total fatty acids)	Linear Regression Equation	r[1]	p<[2]
Activity at 4 nmol Substrate[3]	18:0 (phosphatidylcholine)	y= 9.50x- 77.7	0.41	0.002
	18:1ω7 (cardiolipin)	y= 13.3 x- 12.2	0.31	0.02
	18:2ω6 (phosphatidylcholine)	y=-24.0 x+404	-0.53	0.0005
	20:4ω6 (phosphatidylcholine)	y= 8.47x- 42.5	0.36	0.006
Velocity[4]	18:1ω7 (cardiolipin)	y= 3.51x+ 30.7	0.46	0.0005
	18:2ω6 (phosphatidylcholine)	y=- 5.32x+ 93.2	-0.47	0.0004
	20:4ω6 (phosphatidylcholine)	y= 1.90x- 7.07	0.30	0.02

[1] r, regression correlation coefficient.
[2] p<, significance of correlation.
[3] Values are given for linear regression over all diet treatment groups between y, carnitine palmitoylcarnitine translocase activity at 4 nmol substrate (pmol/min./mg prot.) versus x, liver mitochondrial membrane phospholipid fatty acid levels, (% phospholipid total fatty acids), n=48.
[4] Values are given for linear regression over all diet treatment groups between y, carnitine palmitoylcarnitine translocase velocity (pmol/min./mg prot./nmol palmitoylcarnitine) determined from activity versus palmitoylcarnitine substrate concentration plots versus x, liver mitochondrial membrane phospholipid fatty acid levels (% phospholipid total fatty acids), n=48.

Table 18. Regression Equations Representing the Relationship Between Liver Mitochondrial Membrane Phospholipid Fatty Acid Levels on Carnitine Palmitoyltransferase-1 Function

Function	Fatty Acid (% phospholipid total fatty acids)	Linear Regression Equation	r[1]	p<[2]
Activity at 40 nmol Substrate	18:0 (phosphatidylethanolamine)	y=-12.8 x+997	-0.32	0.01
	Total MUFA[4] (phosphatidylcholine)	y= 22.2 x+378	0.35	0.008
Km[5]	16:0 (phosphatidylcholine)	y=- 6.05x+203	-0.33	0.009
	Total MUFA (total phospholipids)	y=-10.4x+220	-0.35	0.006
	Total PUFA[4] (cardiolipin)	y= 2.89x- 94.0	0.34	0.008
	P/S[4] (total phospholipids)	y=117 x- 48.7	0.32	0.01

[1] r, regression correlation coefficient.
[2] p<, significance of correlation.
[3] Values are given for linear regression over all diet treatment groups between y, carnitine palmitoyltransferase-1 activity at 40 nmol substrate (pmol/min./mg prot.) versus x, liver mitochondrial membrane phospholipid fatty acid levels (% phospholipid total fatty acids), n=48.
[4] Abbreviations used: MUFA = total monounsaturated fatty acids; PUFA = total polyunsaturated fatty acids; P/S = polyunsaturated to saturated fatty acid ratio.
[5] Values are given for linear regression over all diet treatment groups between y, carnitine palmitoyltransferase-1 Km (uM) determined from Eadie-Hofstee plots versus x, liver mitochondrial membrane phospholipid fatty acid levels (% phospholipid total fatty acids), n=48.

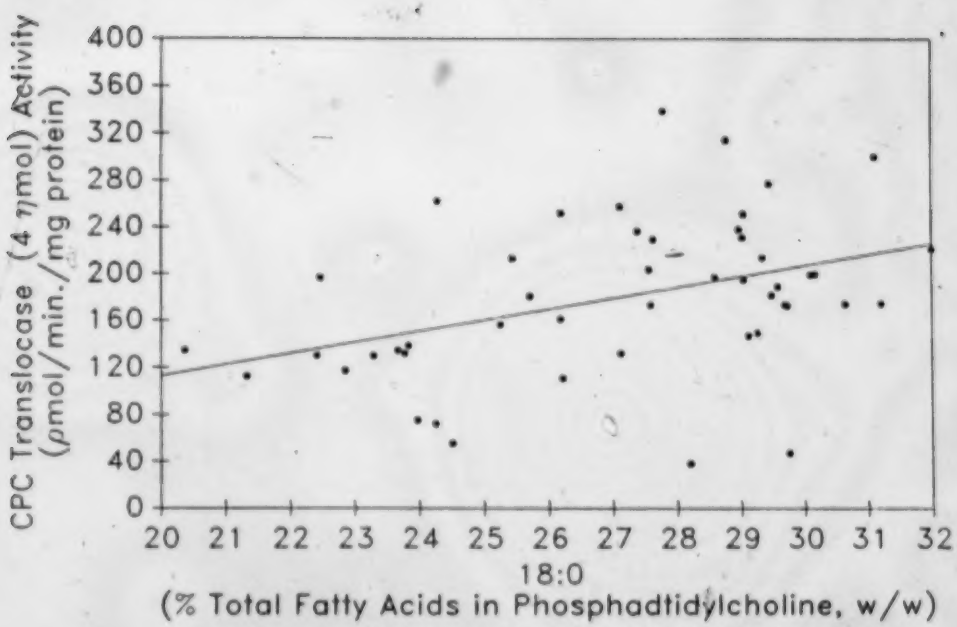

Figure 3. Regression Line Representing the Relationship Between Hepatic Mitochondrial Membrane Phosphatidylcholine 18:0 Levels and Carnitine Palmitoylcarnitine Translocase Activity at 4 nmol Substrate Concentration. Data from individual animals from each dietary treatment group were used to construct the regression line $y = 9.50x - 77.7$ ($r=0.41$, $p<0.002$, $n=48$).

increasing carnitine palmitoylcarnitine translocase activity at 4 nmol substrate concentration (table 17). Decreasing phosphatidylcholine 18:2ω6 levels correlated with increasing carnitine palmitoylcarnitine translocase activity at 4 nmol substrate concentration ($r=-0.53$, $p<0.0005$, figure 4). Decreasing 18:2ω6 levels of phosphatidylcholine ($r=-0.47$, $p<0.0004$) and phosphatidylethanolamine ($r=-0.31$, $p<0.02$, results not shown) correlated with increasing carnitine palmitoylcarnitine translocase velocity. Increasing 20:4ω6 levels of phosphatidylcholine correlated with increasing carnitine palmitoylcarnitine translocase activity at 4 nmol substrate concentration ($r=0.36$, $p<0.006$, figure 5) and increasing carnitine palmitoylcarnitine translocase velocity ($r=0.30$, $p<0.02$). Increasing 18:1ω7 levels of cardiolipin correlated with increasing carnitine palmitoylcarnitine translocase activity at 4 nmol substrate concentration ($r=0.31$, $p<0.02$, figure 6) and increasing carnitine palmitoylcarnitine translocase velocity ($r=0.46$, $p<0.0005$). Increasing 18:1ω7 levels of membrane total phospholipid also correlated with increasing carnitine palmitoylcarnitine translocase velocity ($r=0.41$, $p<0.002$, results not shown).

(b) Carnitine Palmitoyltransferase-1

Increasing mitochondrial membrane 18:1 ω9 levels of phosphatidylcholine ($r=0.32$, $p<0.01$, results not shown) and increasing total monounsaturated fatty acids levels of phosphatidylcholine ($r=0.35$, $p<0.008$) correlated with increasing carnitine palmitoyltransferase-1 activity at 40 nmol substrate concentration

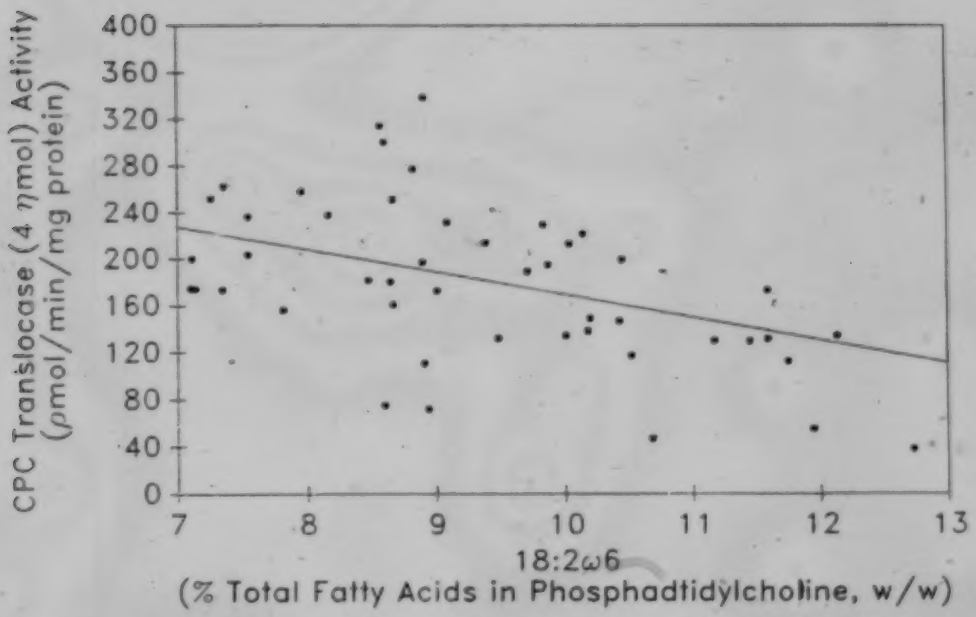

Figure 4. Regression Line Representing the Relationship Between Hepatic Mitochondrial Membrane Phosphatidylcholine 18:2w6 Levels and Carnitine Palmitoylcarnitine Translocase Activity at 4 nmol Substrate Concentration. Data from individual animals from each dietary treatment group were used to construct the regression line $y = -24.0x+404$ ($r=-0.53$, $p<0.0005$, $n=48$).

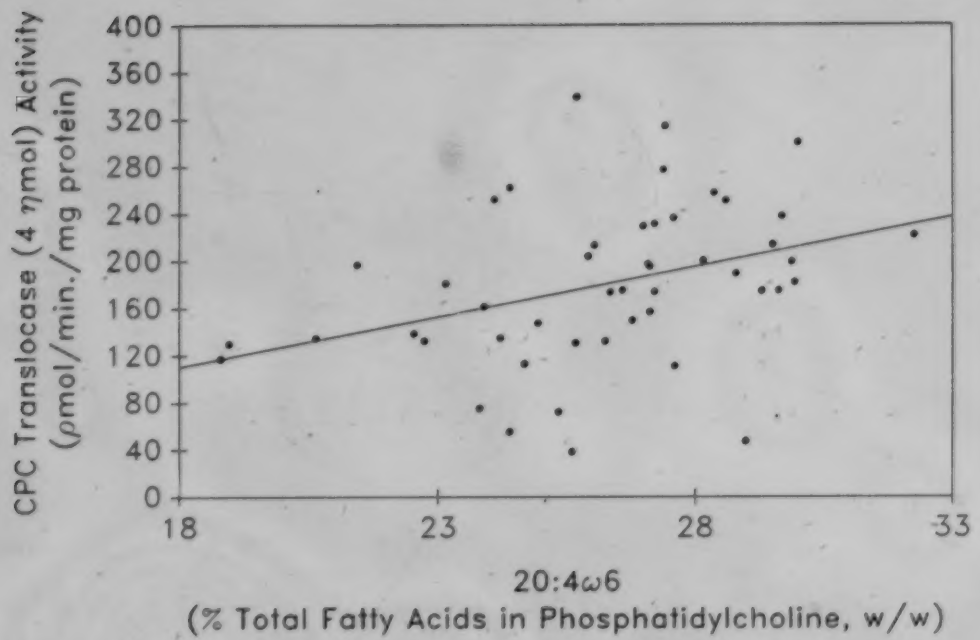

Figure 5. Regression Line Representing the Relationship Between Hepatic Mitochondrial Membrane Phosphatidylcholine 20:4w6 Levels and Carnitine Palmitoylcarnitine Translocase Activity at 4 nmol Substrate Concentration. Data from individual animals from each dietary treatment group were used to construct the regression line y= 8.47x-42.5 ($r=0.36$, $p<0.006$, $n=48$).

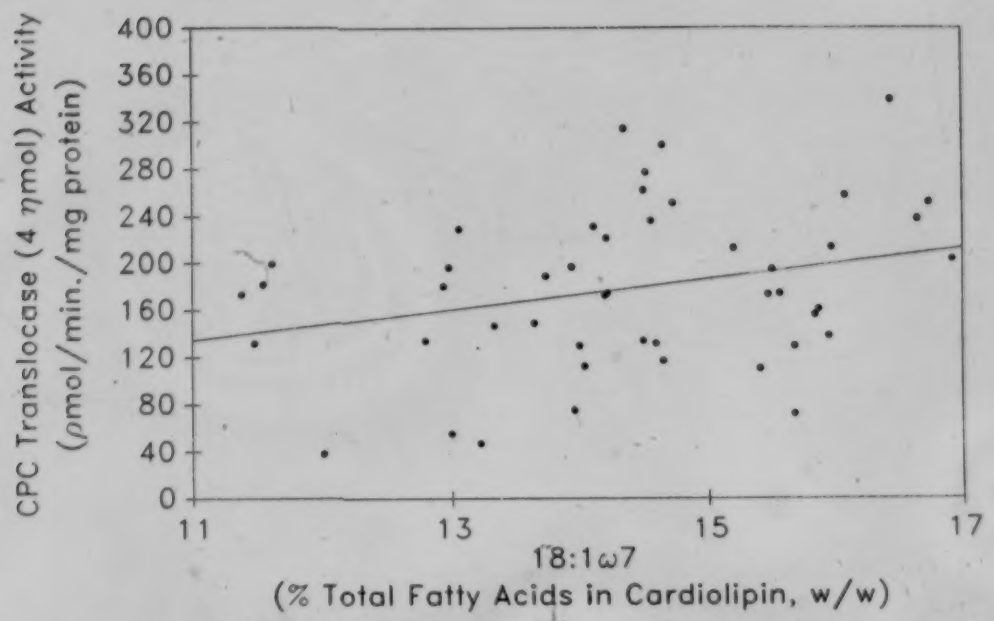

Figure 6. Regression Line Representing the Relationship Between Hepatic Mitochondrial Membrane Cardiolipin 18:1w7 Levels and Carnitine Palmitoylcarnitine Translocase Activity at 4 nmol Substrate Concentration. Data from individual animals from each dietary treatment group were used to construct the regression line $y = 13.3x - 12.2$ ($r=0.31$, $p<0.02$, $n=48$).

(table 18). Increasing 18:1ω9 ($r=-0.28$, $p<0.03$), 18:1ω7 ($r=-0.24$, $p<0.05$), 16:1 ($r=-0.29$, $p<0.02$) and total monounsaturated fatty acid ($r=-0.27$, $p<0.03$) levels of phosphatidylcholine correlated with decreasing carnitine palmitoyltransferase-1 Km (results not shown). Increasing 18:1ω9 ($r=-0.26$, $p<0.04$), 18:1ω7 ($r=-0.23$, $p<0.05$), 16:1 ($r=-0.34$, $p<0.009$) and total monounsaturated fatty acid ($r=-0.26$, $p<0.03$) levels of cardiolipin correlated with decreasing carnitine palmitoyltransferase-1 Km (results not shown). Increasing 18:1ω9 ($r=-0.36$, $p<0.01$), 18:1ω7 ($r=-0.31$, $p<0.01$), and total monounsaturated fatty acid ($r=-0.35$, $p<0.006$, figure 7) levels of total phospholipid correlated with decreasing carnitine palmitoyltransferase-1 Km. Increasing 16:1 ($r=-0.32$, $p<0.01$) levels of phosphatidylethanolamine also correlated with decreasing carnitine palmitoyltransferase-1 Km (results not shown). Increasing 18:0 levels of phosphatidylethanolamine correlated with decreasing carnitine palmitoyltransferase-1 activity at 40 nmol substrate concentration ($r=-0.32$, $p<0.01$). Increasing 16:0 levels of phosphatidylcholine ($r=-0.33$, $p<0.009$, figure 8), phosphatidylethanolamine ($r=-0.28$, $p<0.03$, results not shown) and cardiolipin ($r=-0.37$, $p<0.005$), results not shown) correlated with decreasing carnitine palmitoyltransferase-1 Km. Increasing total saturated fatty acid levels of cardiolipin correlated with decreasing carnitine palmitoyltransferase-1 Km ($r=-0.30$, $p<0.02$, results not shown). Increasing 18:2ω6 content of phosphatidylcholine ($r=0.32$, $p<0.01$) and cardiolipin ($r=0.31$, $p<0.02$) correlated with increasing carnitine palmitoyltransferase-1 (40 nmol) activity (results not shown). Increasing total polyunsaturated ω6

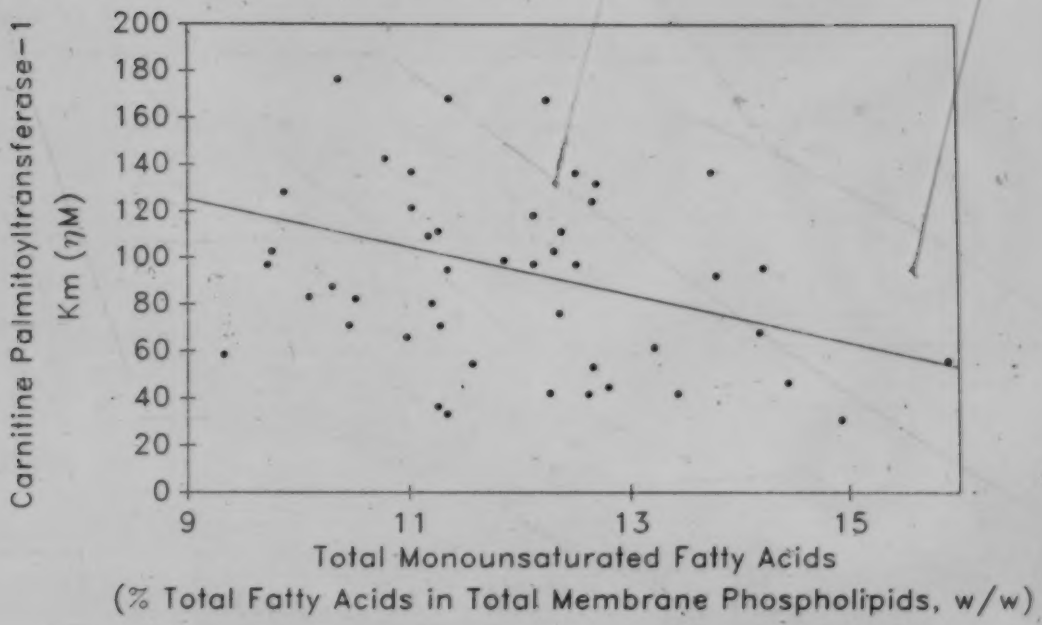

Figure 7. Regression Line Representing the Relationship Between Hepatic Mitochondrial Membrane Total Phospholipid Total Monounsaturated Fatty Acid Levels and Carnitine Palmitoyltransferase-1 Km. The Km of carnitine palmitoyltransferase-1 for individual animals was determined from Eadie-Hofstee plots. Data from individual animals from each dietary treatment group were used to construct the regression line $y = -10.4x+220$ ($r=-0.35$, $p<0.006$, $n=48$).

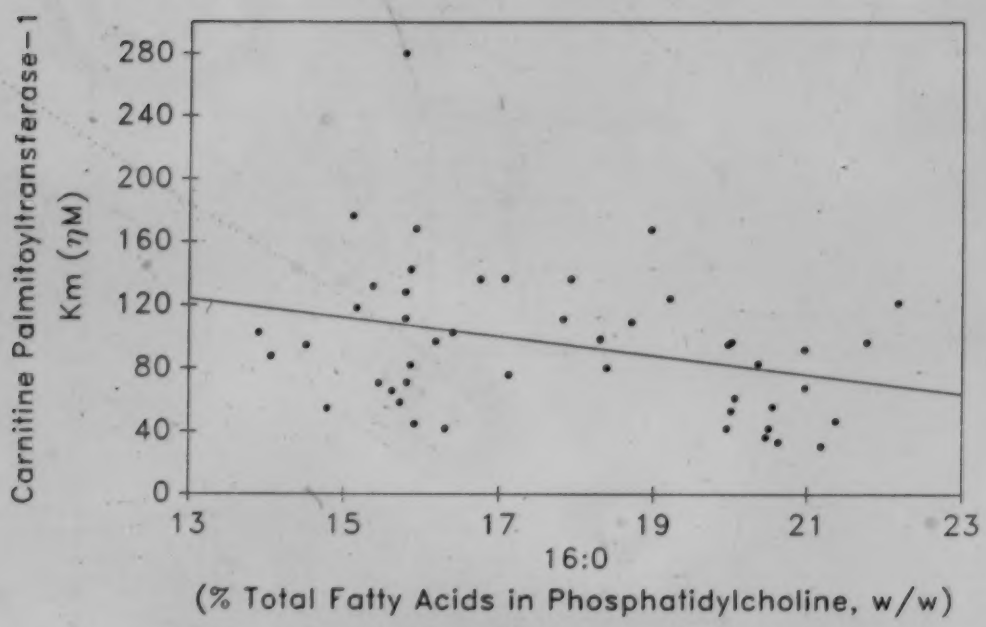

Figure 8. Regression Line Representing the Relationship Between Hepatic Mitochondrial Membrane Phosphatidylcholine 16:0 Levels and Carnitine Palmitoyltransferase-1 Km. The Km of carnitine palmitoyltransferase-1 for individual animals was determined from Eadie-Hofstee plots. Data from individual animals from each dietary treatment group were used to construct the regression line $y = -6.05x+203$ ($r=-0.33$, $p<0.009$, $n=48$).

fatty acid levels of phosphatidylcholine ($r=0.38$, $p<0.004$), phosphatidylethanolamine ($r=0.25$, $p<0.04$), and cardiolipin ($r=0.35$, $p<0.008$) correlated with increasing carnitine palmitoyltransferase-1 Km (results not shown). Increasing total polyunsaturated $\omega 3$ and $\omega 6$ fatty acid levels of phosphatidylcholine ($r=0.31$, $p<0.01$, results not shown), phosphatidylethanolamine ($r=0.29$, $p<0.02$, results not shown), and cardiolipin ($r=0.34$, $p<0.008$, figure 9), correlated with increasing carnitine palmitoyltransferase-1 Km. Increasing polyunsaturated to saturated fatty acid ratios of phosphatidylcholine ($r=0.34$, $p<0.009$, results not shown), phosphatidylethanolamine ($r=0.28$, $p<0.02$, results not shown), cardiolipin ($r=0.33$, $p<0.01$, results not shown), and total phospholipids ($r=0.32$, $p<0.01$) also correlated with increasing carnitine palmitoyltransferase-1 Km.

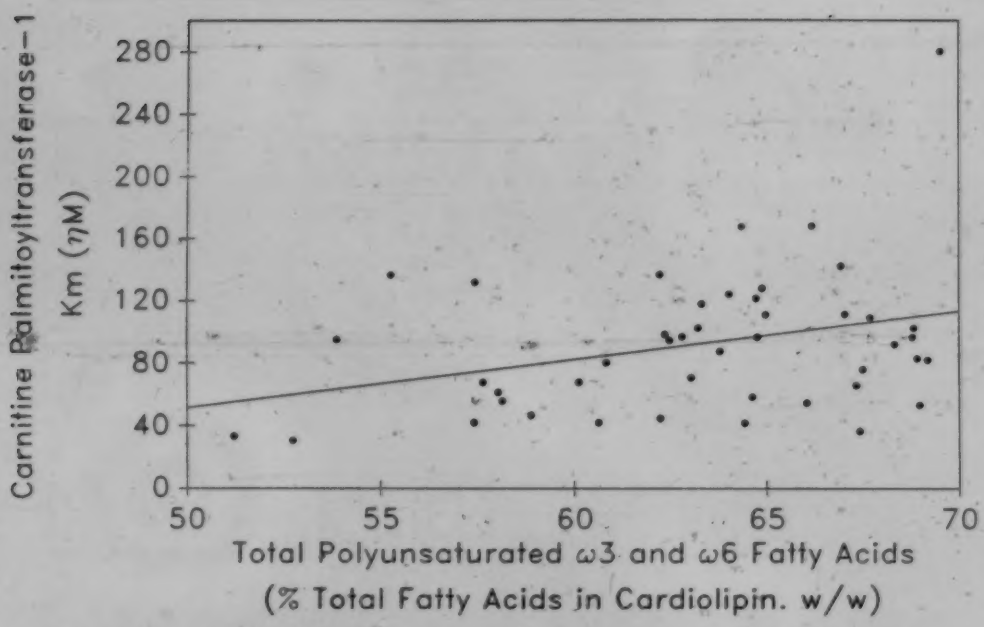

Figure 9. Regression Line Representing the Relationship Between Hepatic Mitochondrial Membrane Cardiolipin Total Polyunsaturated Fatty Acid Levels and Carnitine Palmitoyltransferase-1 Km. The Km of carnitine palmitoyltransferase-1 for individual animals was determined from Eadie-Hofstee plots. Data from individual animals from each dietary treatment group were used to construct the regression line $y = 2.89x - 94.0$ ($r=0.34$, $p<0.008$, $n=48$).

DISCUSSION

Previous studies, such as those examining ATPases (4,5,6,), adenylate cyclase (6,7), and transporter enzymes (8-11) have demonstrated that membrane fluidity, altered by changing phospholipid fatty acid unsaturation levels, affected membrane protein function. Previous studies have demonstrated that alterations in membrane lipid composition by dietary fat were also associated with altered membrane protein function (8,9,12-15). The experiment presented in this thesis extends current knowledge by examining the hypothesis that changes in dietary fatty acid composition alter liver mitochondrial membrane composition and affects carnitine dependent fatty acid transport into liver mitochondria.

I. EFFECT OF DIET TREATMENT ON LIVER MITOCHONDRIAL MEMBRANE PHOSPHOLIPID CONTENT

Fat content (15) and fatty acid composition of diet (33,34) have previously been shown to alter membrane phospholipid distribution. In the present study, it was hypothesized that increasing dietary unsaturated fatty acid levels would increase major liver mitochondrial membrane unsaturated fatty acid containing phospholipids, such as cardiolipin. Results of the present study were consistent with this hypothesis. In this regard, mitochondrial membrane cardiolipin levels increased in rats fed high fat diets (table 3). Mitochondrial membrane cardiolipin contained more than 30% of the membrane total unsaturated (total monounsaturated and total polyunsaturated $\omega 3$ and $\omega 6$) fatty

acids and was comprised of greater than 91% unsaturated fatty acids. Because high fat diets contained proportionately more unsaturated fatty acids than did low fat diets and because cardiolipin contained the highest proportion of unsaturated fatty acids, increases in membrane cardiolipin levels in rats fed high fat diets may have been due to preferential incorporation of dietary unsaturated fatty acids into cardiolipin phospholipids. Cardiolipin comprises greater than 46% of membrane total acidic phospholipids. In this regard, increasing membrane cardiolipin levels in rats fed high fat diets were likely responsible for increases observed in mitochondrial membrane total acidic phospholipid levels in rats fed high fat diets.

Increases in individual neutral phospholipid (phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin) levels (ug/mg protein), although not significant, were observed in hepatic mitochondrial membranes of rats fed high fat diets. Significant decreases in total neutral phospholipid levels (% total phospholipids) were observed in mitochondrial membranes of rats fed high fat diets compared to rats fed low fat diets (table 4). This was due to greater increases in acidic phospholipid levels (% total phospholipids) than in neutral phospholipid levels (% total phospholipids) in mitochondrial membranes of rats fed high fat diets.

In the present study, total phospholipid content (ug/mg mitochondrial protein) of liver mitochondrial membranes of all diet groups, was 28% to 45% higher than previously reported (35, table 3). This difference in total phospholipid content could be due to age and strain differences between the experimental rats examined.

Mitochondrial membrane total phospholipid levels were not significantly altered by either dietary fat levels or dietary fatty acid composition. This may have been due to variation of approximately 15 to 23% in total phospholipid levels determined within each diet group. Dietary polyunsaturated to saturated fatty acid ratio did not significantly alter mitochondrial membrane phospholipid distribution. But a trend towards higher mitochondrial membrane levels of all phospholipids, except cardiolipin whose level decreased, were observed in rats fed diets with low polyunsaturated to saturated fatty acid ratios.

Robblee and Clandinin (15) fed rats diets providing 40% or 15% of calories as fat, but with polyunsaturated to unsaturated fatty acid ratios of 0.25 or 2.0. They found both cardiolipin and phosphatidylcholine significantly increased in cardiac mitochondrial membranes in rats fed high fat diets. Robblee and Clandinin (15) also found that dietary polyunsaturated to saturated fatty acid ratio had no significant effects on membrane phospholipid distribution. Feeding diets more extreme in polyunsaturated to saturated fatty acid ratios, such as 0.25 versus 3.0, or longer duration of feeding, may have produced more significant differences in mitochondrial membrane phospholipid distribution.

II. EFFECT OF DIET ON LIVER MITOCHONDRIAL MEMBRANE PHOSPHOLIPID FATTY ACID COMPOSITION

The fatty acid composition of liver mitochondrial membrane phospholipid classes were different from one another (tables 5 to 11). This is in agreement with previous studies (15,36,37). Previous

studies also demonstrated that dietary fat levels (15) and fatty acid composition (14,15,36,37,38) alter membrane phospholipid fatty acid composition. In the present study, it was hypothesized that increasing dietary unsaturated fatty acid content would increase liver mitochondrial membrane phospholipid levels of unsaturated fatty acids. Results of the present study were consistent with this hypothesis. In this regard, total polyunsaturated ω3 and ω6 fatty acid content in liver mitochondrial membrane phospholipids were increased by feeding rats diets of increasing fat and polyunsaturated fatty acid content.

Rats, being homeotherms, are obliged to maintain a narrow range of temperature dependent fluidity in their membranes for optimal membrane protein function (39). Fluidity is partially modulated by fatty acid composition of the membrane. Transition temperatures and enthalpy changes are lower for phospholipids containing unsaturated fatty acids than for phospholipids containing saturated fatty acids of the same chain length (40). This may be interpreted to imply that fluidity of mitochondrial membranes are increased as membrane phospholipid unsaturated fatty acid content is increased. In the present study, total saturated fatty acid levels of mitochondrial membrane phospholipids were slightly decreased by high dietary fat levels and by high dietary polyunsaturated to saturated fatty acid levels (table 12). Total polyunsaturated ω3 and ω6 fatty acid content of membrane phospholipids was significantly increased by feeding high fat diets and by feeding diets with high polyunsaturated to saturated fatty acid ratios (tables 5 to 12). It is, therefore not surprising that increased polyunsaturated to saturated fatty acid ratio of most

mitochondrial membrane phospholipids were observed in rats fed diets of high fat content or of high polyunsaturated to saturated fatty acid ratios (tables 5 to 11).

Mitochondrial membrane total phospholipid levels were not significantly different between dietary treatment groups (table 3). Total unsaturated fatty acid content of phospholipids remained relatively constant (table 12). Therefore, observed increases in mitochondrial membrane phospholipid total polyunsaturated 3 and 6 fatty acid levels without changing phospholipid total unsaturated fatty acid levels were achieved by observed decreases in phospholipid total monounsaturated fatty acid levels. This observed pattern of fatty acid manipulation by diet was likely brought about by homeoviscous adaptation. Homeoviscous adaptation is the action taken by an organism to maintain constant membrane lipid fluidity (39).

Diets more extreme in fat content and polyunsaturated fatty acid levels, such as polyunsaturated to saturated fatty acid ratios of 0.25 and 3.0 or longer duration of feeding, might have produced more significant differences in mitochondrial membrane phospholipid fatty acid composition.

III. EFFECT OF DIET ON HEPATIC CARNITINE PALMITOYLCARNITINE TRANSLOCASE AND CARNITINE PALMITOYLTRANSFERASE-1 FUNCTIONS

Alterations in membrane protein function due to modifications in lipid environment by dietary fat have been reported for various membranes, including rat mitochondrial membrane (14,15,41,42,43). In the present study, the general hypothesis that dietary fatty acid

composition affects carnitine dependent fatty acid transport into liver mitochondria was examined by observing the effect of increasing dietary fat levels and polyunsaturated fatty acid ratios on carnitine palmitoylcarnitine translocase (table 13) and carnitine palmitoyltransferase-1 functions (table 14).

(a) Carnitine Palmitoylcarnitine Translocase

Effects of dietary fat level and dietary polyunsaturated to saturated fatty acid ratio appeared to produce inconsistencies in carnitine palmitoylcarnitine translocase functions (table 13). No significant effect of diet on carnitine palmitoylcarnitine translocase activity was demonstrated by repeated measures of analysis of variance. High dietary fat levels significantly increased carnitine palmitoylcarnitine translocase activity at 4 nmol substrate concentration and no significant effect of diet on carnitine palmitoylcarnitine translocase velocity was observed. Translocase velocity was determined as the slope of linear regression lines fitted to plots of carnitine palmitoylcarnitine translocase activity versus substrate concentration. Activity of carnitine palmitoylcarnitine translocase did not always increase linearly with increasing substrate concentration. Slopes of regression lines were non-uniformly lowered by leveling off of carnitine palmitoylcarnitine translocase activity at higher substrate concentrations. Therefore, slopes of the linear regression line did not always accurately represent the true velocity of carnitine palmitoylcarnitine translocase. This may explain why significant differences in carnitine palmitoylcarnitine translocase

activity at 4 nmol substrate concentration but no significant differences in carnitine palmitoylcarnitine translocase velocities were observed in rats fed high fat diets. Measured parameters of carnitine palmitoylcarnitine translocase function demonstrated a trend towards increasing activity in rats fed high fat with low polyunsaturated to saturated fatty acid ratio diets. High within group variation due to experimental design likely prevented all observed differences from being significant.

(b) Carnitine Palmitoyltransferase-1

Observed effects of dietary fat on carnitine palmitoyltransferase-1 functions were also inconsistent. Repeated measures of analysis of variance demonstrated a significant increase in carnitine palmitoyltransferase-1 activity in rats fed high fat diets. But, dietary fat level had no significant effects on carnitine palmitoyltransferase-1 activity at 40 nmol substrate concentration. Carnitine palmitoyltransferase-1 activity at 40 nmol substrate concentration in rats fed low fat, low polyunsaturated to saturated fatty acid ratio diets were higher than would have been predicted based on patterns of carnitine palmitoyltransferase-1 activity determined from repeated measures analysis of variance. The reasons for this were not clear. Low dietary polyunsaturated to saturated fatty acid ratios significantly increased carnitine palmitoyltransferase-1 activity at 40 nmol substrate concentration. But, dietary polyunsaturated to saturated fatty acid ratios had no significant effect on total carnitine palmitoyltransferase-1 activity demonstrated by repeated

measures of analysis of variance. Total carnitine palmitoyltransferase-1 activity demonstrated a trend towards increasing activity with decreasing dietary polyunsaturated to saturated fatty acid ratios, but high within group variation due to experimental design may have prevented a significant difference from being demonstrated. Results of the present study demonstrated that dietary fat level and fatty acid composition significantly altered carnitine palmitoyltransferase-1 activity. The Km of carnitine palmitoyltransferase-1 was not significantly altered by dietary fat levels or fatty acid composition, therefore, increases in activity observed may be due to increases in the number of active carnitine palmitoyltransferase-1 molecules rather than actual increases in carnitine palmitoyltransferase-1 activity. Further research is required in this area.

Upon examination of the present data of carnitine palmitoylcarnitine translocase and carnitine palmitoyltransferase-1 function, trends consistent with significant data are apparent (tables 11 and 12). High fat diets generally increased carnitine palmitoylcarnitine translocase and carnitine palmitoyltransferase-1 activity, and high dietary polyunsaturated to saturated fatty acid ratios generally decreased carnitine palmitoylcarnitine translocase and carnitine palmitoyltransferase-1 activity levels. The significance and implications of these observations are addressed in the general discussion section. Diets more extreme in fat content and fatty acid composition, such as polyunsaturated to saturated fatty acid ratios of 0.25 and 3.0, or longer duration of feeding may have produced more

significant differences in carnitine palmitoyltransferase-1 and carnitine palmitoylcarnitine translocase functions between diet groups.

IV. RELATIONSHIP BETWEEN LIVER MITOCHONDRIAL MEMBRANE PHOSPHOLIPID DISTRIBUTION AND CARNITINE PALMITOYLCARNITINE TRANSLOCASE AND CARNITINE PALMITOYLTRANSFERASE-1 FUNCTIONS

In the present study, it was hypothesized that increasing levels of unsaturated fatty acid containing liver mitochondrial membrane phospholipids, such as cardiolipin, would increase carnitine palmitoylcarnitine translocase and carnitine palmitoyltransferase-1 functions. In this regard, it was determined if relationships existed between mitochondrial membrane phospholipid levels and carnitine palmitoylcarnitine translocase (table 15) or carnitine palmitoyltransferase-1 activity (table 16). Results of the present study were consistent with this hypothesis.

(a) Carnitine Palmitoylcarnitine Translocase

Increasing carnitine acylcarnitine translocase activity due to increasing membrane incorporation of cardiolipin has been demonstrated for reconstituted carnitine acylcarnitine translocase enzyme (20). Results of the present study also demonstrate that increasing liver mitochondrial membrane cardiolipin levels correlates with both increasing carnitine palmitoylcarnitine translocase activity at 4 nmol substrate concentration and carnitine palmitoylcarnitine translocase velocity (table 15). Cardiolipin levels might, therefore, function in vivo to modulate carnitine palmitoylcarnitine translocase activity.

Results obtained also suggest that of all acidic phospholipids, cardiolipin exhibited the strongest association with carnitine palmitoylcarnitine translocase activity.

Noel et al (21) found that carnitine acylcarnitine translocase reconstituted in lipid vesicles required addition of asolectin (65% phospholipid) for adequate activity. Asolectin is soyabean lecithin which is composed primarily of phosphatidylcholine. Phosphatidylethanolamine is the usual contaminant. Increasing carnitine palmitoylcarnitine translocase activity with increasing asolectin incorporation into reconstituted membranes supports the present observation that increasing mitochondrial membrane phosphatidylcholine and phosphatidylethanolamine levels correlated with increasing carnitine palmitoylcarnitine translocase velocity and activity at 4 nmol substrate concentration (table 15). Because phosphatidylcholine and phosphatidylethanolamine comprised the majority of membrane ~~total~~ neutral phospholipids, increases observed in the velocity of carnitine palmitoylcarnitine translocase with increasing total neutral phospholipid levels would be expected.

It was observed that as mitochondrial membrane neutral to acidic phospholipid ratios increased carnitine palmitoylcarnitine translocase velocity and activity at 4 nmol substrate concentration decreased. If neutral phospholipids exhibited a greater effect on carnitine palmitoylcarnitine translocase velocity and activity at 4 nmol substrate concentration than cardiolipin levels, then total neutral to total acidic phospholipid ratios would have increased as carnitine palmitoylcarnitine translocase velocity and activity at 4 nmol substrate concentration increased. This suggested that mitochondrial

membrane cardiolipin levels had a greater association with carnitine palmitoylcarnitine translocase velocity and activity at 4 nmol substrate concentration than mitochondrial membrane neutral phospholipid levels. Levels of cardiolipin may have had the strongest association with carnitine palmitoylcarnitine translocase activity.

Increasing carnitine palmitoylcarnitine translocase velocity correlated with increasing mitochondrial membrane total acidic and total neutral phospholipid levels. Acidic phospholipids comprised 44% of mitochondrial membrane phospholipids, while neutral phospholipids comprised the remaining 56%. It is therefore, not surprising that increasing carnitine palmitoylcarnitine translocase velocity also correlated with increasing membrane total phospholipid levels. Although membrane total phospholipid levels were not significantly different between dietary treatments, high within dietary treatment group variation made it possible to construct scatterplots of membrane total phospholipid levels versus carnitine palmitoylcarnitine translocase velocity and activity at 4 nmol substrate concentration.

(b) Carnitine Palmitoyltransferase-1

Increasing carnitine palmitoyltransferase-1 activity with increasing mitochondrial membrane cardiolipin level has previously been demonstrated for reconstituted carnitine palmitoyltransferase-1 (16,17). In the present study, it was observed that both carnitine palmitoyltransferase-1 activity at a substrate concentration of 40 nmol and the Km for carnitine palmitoyltransferase-1 increased as mitochondrial membrane cardiolipin levels increased (table 16).

The total level of phospholipids with single net negative charges on their head groups (phosphatidylinositol, phosphatidylserine and phosphatidylglycerol) had no significant affect on carnitine palmitoyltransferase-1 activity at 40 nmol substrate concentration. Increasing total mitochondrial membrane levels of phosphatidylinositol, phosphatidylserine and phosphatidylglycerol correlated with decreasing Km for carnitine palmitoyltransferase-1. It is not clear from this experiment whether or not liver mitochondrial membrane phospholipids with a single net negative charge on their head group affected carnitine palmitoyltransferase-1 activity. Increasing Km of carnitine palmitoyltransferase-1 correlated with increasing ratios of membrane total neutral to total levels of phosphatidylinositol, phosphatidylserine and phosphatidylglycerol. Therefore, decreases in carnitine palmitoyltransferase-1 Km due to increased levels of membrane phospholipids with single net negative charges may be modulated by increasing membrane total neutral phospholipid levels. Results obtained were inconclusive and further research is required in this area.

Woldegiorgis et al (16) and Pande et al (17) found a significant increase in reconstituted carnitine palmitoyltransferase-1 activity upon addition of phosphatidylcholine to lipid vesicles. The present study demonstrated that increasing mitochondrial membrane phosphatidylcholine, phosphatidylethanolamine and neutral phospholipid levels correlated with increasing carnitine palmitoyltransferase-1 activity at 40 nmol substrate concentration and Km (table 16).

Sire et al found D-galactoseamine treatment (18), which decreased

membrane total phospholipid levels, also decreased carnitine palmitoyltransferase-1 activity, and that clofibrate treatment (19), which increased membrane total phospholipid levels, also increased carnitine palmitoyltransferase-1 activity. These observations support the present finding that carnitine palmitoyltransferase-1 activity at 40 nmol substrate concentration increased as mitochondrial membrane total phospholipid levels increased (table 16). Increasing carnitine palmitoyltransferase-1 activity at 40 nmol substrate concentration with increasing mitochondrial membrane total phospholipid levels, were likely attributable to increasing mitochondrial membrane total acidic and total neutral phospholipid content. Because cardiolipin levels affected both carnitine palmitoyltranslocase Km and activity at 40 nmol substrate concentration, liver mitochondrial membrane cardiolipin levels likely had the strongest influence on carnitine palmitoyltransferase-1 activity than any other individual mitochondrial membrane phospholipid. Although significant relationships between membrane phospholipid levels and carnitine palmitoyltransferase-1 activity existed, correlations observed were very weak. More research is required in this area to determine the extent of membrane phospholipid level relationship with carnitine palmitoyltransferase-1 activity.

V. RELATIONSHIP BETWEEN LIVER MITOCHONDRIAL MEMBRANE PHOSPHOLIPID FATTY ACID COMPOSITION AND CARNITINE PALMITOYLCARNITINE TRANSLOCASE AND CARNITINE PALMITOYLTRANSFERASE-1 FUNCTIONS

In the present study, it was hypothesized that increasing liver

mitochondrial membrane phospholipid total polyunsaturated fatty acid content would increase carnitine palmitoylcarnitine translocase and carnitine palmitoyltransferase-1 function. In this regard, it was determined whether or not relationships existed between levels of mitochondrial membrane phospholipid fatty acids and activity of carnitine palmitoylcarnitine translocase (table 17) or carnitine palmitoyltransferase-1 (table 18). Results of the present study were consistent with the hypothesis.

(a) Carnitine Palmitoylcarnitine Translocase

Levels of 18:0, 18:1ω7, 18:2ω6, and 20:4ω6 in mitochondrial membrane phospholipids correlated with hepatic mitochondrial carnitine palmitoylcarnitine translocase function (table 17). Increasing 18:0 levels in phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol correlated with increasing carnitine palmitoylcarnitine translocase activity at 4 nmol substrate concentration and increasing carnitine palmitoylcarnitine translocase velocity. Increasing phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol levels also correlated with increasing velocity of carnitine palmitoylcarnitine translocase. It is not clear from these results whether mitochondrial membrane phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol levels had a greater influence on carnitine palmitoylcarnitine translocase function than did 18:0 levels of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol or whether 18:0 levels of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol had a greater

influence on carnitine palmitoylcarnitine translocase function than phosphatidylcholine, phosphatidylethanolamine, or phosphatidylinositol levels. More research is required in this area.

A significant proportion (14%, w/w) of liver mitochondrial membrane cardiolipin fatty acids was comprised of 18:1ω 7. As previously discussed for 18:0 of mitochondrial membrane phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol, it is not clear whether the correlation between 18:1ω7 and carnitine palmitoylcarnitine translocase activity was due to association of 18:1ω7 with cardiolipin or not. The gas chromatographic analysis used to determine the fatty acid composition of membrane phospholipids was not designed to specifically resolve 18:1ω9 and 18:1ω7 fatty acid levels. It is possible that 18:1ω9 and 18:1ω7 separations were not complete. Therefore, the significance of the observed correlation of carnitine palmitoylcarnitine translocase activity with membrane phospholipid 18:1ω7 levels is questionable.

It was observed that increases in carnitine palmitoylcarnitine translocase velocity and activity at 4 nmol substrate concentration correlated with increases in 20:4ω6 levels and decreases in carnitine palmitoylcarnitine translocase velocity and activity at 4 nmol substrate concentration correlated with decreasing 18:2 ω6 levels. Enzymes of fatty acid chain elongation and desaturation are capable of converting 18:2 ω6 to 20:4ω 6 (44). Recent studies have shown that enzymes of fatty acid chain shortening and saturation may convert 20:4 ω6 to 18:2 ω 6 (45). Modulation of mitochondrial membrane phospholipid 18:2ω6 and 20:4ω6 levels might, therefore, be an in vivo

control mechanism of carnitine palmitoylcarnitine translocase activity.

(b) Carnitine Palmitoyltransferase-1

Both increasing Km of carnitine palmitoyltransferase-1 and activity at 40 nmol substrate concentration correlated with decreasing levels of saturated fatty acids (table 18). Similar to membrane total phospholipid levels, Km of carnitine palmitoyltransferase-1 were not significantly different between dietary treatment groups. Because large variations in Km of carnitine palmitoyltransferase-1 were present within each dietary treatment group, it was possible to construct scatterplots of mitochondrial membrane phospholipid saturated fatty acid levels versus carnitine palmitoyltransferase-1 Km.

Contradictions were observed between the association of mitochondrial membrane phospholipid monounsaturated fatty acids with carnitine palmitoyltransferase-1 activity at 40 nmol substrate concentration and Km. Increasing carnitine palmitoyltransferase-1 activity at 40 nmol substrate concentration correlated with increasing phospholipid total monounsaturated fatty acids of phosphatidylethanolamine. Increasing carnitine palmitoyltransferase-1 Km correlated with decreasing phospholipid total monounsaturated fatty acid levels of phosphatidylcholine, phosphatidylethanolamine, cardiolipin and membrane total phospholipids. Because of overwhelming numbers and consistency, carnitine palmitoyltransferase-1 Km correlation results might have been more representative of the action of mitochondrial membrane phospholipid monounsaturated fatty acid levels on carnitine palmitoyltransferase-1 function. Further study to

confirm this assumption is required.

Unlike mitochondrial membrane 18:2ω6 fatty acid level influences on carnitine palmitoylcarnitine translocase velocity and activity at 4 nmol substrate concentration, increasing mitochondrial membrane phospholipid 18:2 ω6 levels correlated with increasing carnitine palmitoyltransferase-1 activity at 40 nmol substrate concentration and Km. Increasing mitochondrial membrane phospholipid total polyunsaturated ω3 and ω 6 fatty acid levels correlated with increasing carnitine palmitoyltransferase-1 Km. Increasing mitochondrial membrane phospholipid total saturated fatty acid levels correlated with decreasing carnitine palmitoyltransferase-1 Km. Increasing mitochondrial membrane phospholipid polyunsaturated to saturated fatty acid ratio also correlated with increasing carnitine palmitoyltransferase-1 Km. The above results indicated that mitochondrial membrane phospholipid polyunsaturated fatty acid levels have a stronger association with carnitine palmitoyltransferase-1 Km than mitochondrial membrane saturated fatty acid levels.

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CONCLUSIONS AND GENERAL DISCUSSION

The carnitine fatty acid transport shuttle system is an intramembrane protein system composed of carnitine palmitoyltransferase-1 and carnitine palmitoyltransferase-2 peripheral membrane proteins and a carnitine palmitoylcarnitine translocase integral membrane protein. Because of carnitine palmitoylcarnitine translocase's intimate association with membranes, its activity has the potential to be modulated by membrane lipid environment. The extent of the association of carnitine palmitoyltransferase-1 and carnitine palmitoyltransferase-2 with membrane lipid is unknown. It is possible that carnitine palmitoyltransferase-1 and carnitine palmitoyltransferase-2 have little direct physical contact with membrane lipids and therefore, may not be strongly affected by altering membrane lipid composition.

It was specifically hypothesized that increasing the unsaturated fatty acid content of dietary fat would increase levels of major unsaturated fatty acid containing phospholipids, such as cardiolipin, and that the unsaturated fatty acid content of phospholipids would also increase. It was also specifically hypothesized that increasing the liver mitochondrial levels of major phospholipids, such as cardiolipin, would increase carnitine palmitoylcarnitine translocase and carnitine palmitoyltransferase-1 functions and that increasing unsaturated fatty acid content of liver mitochondrial membrane phospholipids would increase the activity of carnitine palmitoylcarnitine translocase and carnitine palmitoyltransferase-1. Except for phospholipid levels of 18:2 ω6 which decreased carnitine palmitoylcarnitine translocase

activity, results of the present study are consistent with the above hypotheses.

It was generally hypothesized that altering dietary fatty acid composition would alter liver mitochondrial membrane composition and would affect carnitine dependent fatty acid transport into liver mitochondria. Carnitine dependent fatty acid transport is regulated by acitivities of carnitine palmitoylcarnitine translocase and carnitine palmitoyltransferase-1. In this regard, high fat diets were observed to increase cardiolipin content of mitochondrial membranes. Increasing cardiolipin levels correlated with increasing carnitine palmitoyl-carnitine translocase and carnitine palmitoyltransferase-1 activities, which was consistent with the general hypothesis. Diets with high polyunsaturated to saturated fatty acid ratios were observed to increase polyunsaturated fatty acid composition of membrane phospholipids, but diets with low polyunsaturated to saturated fatty acid ratios were observed to increase carnitine palmitoylcarnitine translocase and carnitine palmitoyltransferase-1 functions. This was not consistent with the general hypothesis.

It was observed that increases in carnitine palmitoylcarnitine translocase velocity and activity at 4 nmol substrate concentration correlated with increasing phospholipid 20:4 ω 6 levels and that decreasing carnitine palmitoylcarnitine translocase velocity and activity at 4 nmol substrate concentration correlated with increasing phospholipid 18:2 ω 6 levels. Because mitochondrial membrane phospholipid levels, mitochondrial membrane phospholipid fatty acid levels and carnitine palmitoylcarnitine translocase function were not

determined prior to diet treatment, baseline values were not obtained. The dietary fat fed contained large amounts of 18:2ω6 and only trace amounts of 20:4ω6, therefore, it is possible that liver mitochondrial membrane phospholipid 18:2ω6 levels were elevated relative to 20:4ω6 levels. Increasing membrane phospholipid 18:2ω6 levels relative to 20:4ω6 may explain why diets with high polyunsaturated to saturated ratios decreased carnitine palmitoylcarnitine translocase function compared to diets with low polyunsaturated to saturated fatty acid ratios. It was not clear why diets with low polyunsaturated to saturated fatty acid ratios produced increased hepatic carnitine palmitoyltransferase-1 function. Effects of dietary fat on membrane composition did not support the finding that diets with low polyunsaturated to saturated fatty acid ratios increasing carnitine palmitoyltransferase-1 function. Perhaps carnitine palmitoyltransferase-1 has little direct physical contact with membrane lipids, therefore, observed dietary effects on carnitine palmitoyltransferase-1 function may not have been due to liver mitochondrial membrane lipid composition. Regulation of carnitine palmitoyltransferase-1 activity is very complex (1). Hormones (1-5), metabolic inhibitors (6), such malonyl-CoA (7,8), and mitochondrial matrix carnitine levels (9,10,11) have all been shown to influence carnitine palmitoyltransferase-1 function.

NUTRITIONAL IMPLICATIONS

Modulation of carnitine palmitoyltransferase-1 and carnitine palmitoylcarnitine translocase functions through diet may have significant biological implications. Diet treatments used in the present study, were designed to reflect current american dietary fat intakes ranging from diets with high fat and low polyunsaturated to saturated fatty acid ratios to diets with low fat and high polyunsaturated to saturated fatty acid ratios. Diets fed in the present study also followed America Academy of Pediatrics recommendations (1976). Currently the American Heart Association (1986) recommends diets low in fat (approximately 30 %) with polyunsaturated to saturated fatty acid ratios of 1.0. This recommended reduction in saturated fatty acid intake is proposed to reduce the risk of heart disease. The American Academy of Pediatrics (1976) recommends a minimum of 30% of total energy intake be supplied as fat and 3% of total energy intake be supplied as linoleic acid.

Total parenteral nutrition infusions utilizing Nutralipid™ or Intralipid™ as fat sources in neonatal intensive care units are composed of approximately 30% fat (on a per calorie basis) with polyunsaturated to saturated fatty acid ratio of approximately 3.0 (12,13). Nutralipid™ and Intralipid™ are composed of 50% linoleic acid therefore neonates consume 15% of their total energy as linoleic acid. Linoleic acid content of total parenteral nutrition solutions is much greater than linoleic acid content of human breast milk. On average, human milk supplies approximately 50% of total energy as fat and approximately 5% of total energy as linoleic acid (14). Human breast

milk compositon is strongly influenced by maternal dietary intake and, therefore, fat levels and linoleic acid content vary (14). Neonatal total parenteral nutrition formulations are an exaggeration of American Heart Association and American Academy of Pediatrics recommendations with respect to polyunsaturated to saturated fatty acid ratios and linoleic acid content, respectively. Lipid solubility dictates high levels of linoleic fatty acids in Nutralipid™ and Intralipid™. Few studies of the effects of high linoleic fatty acid levels in neonatal nutrition exist in the literature.

Total parenteral infusions in adults utilizing Nutralipid™ or Intralipid™ as fat sources vary widely in fat level, but have polyunsaturated to saturated fatty acid ratios of approximately 3.0. Fat content varies from a minimum of 4% of calories, to meet essential fatty acid requirements, to approximately 50% of calories. The amount of fat administered is calculated using established guidelines (15). Adult total parenteral nutrition formulations are also an exaggeration of the American Heart Association recommendations with respect to polyunsaturated to saturated fatty acid ratios. No detrimental effects or complications of infusing fat emulsions with polyunsaturated to saturated fatty acid ratios of 3.0 have been reported in the literature.

Results of the present study indicated that diets composed of 40% fat with polyunsaturated to saturated fatty acid ratios of 0.35 increased both carnitine palmitoylcarnitine translocase and carnitine palmitoyltransferase function in liver mitochondria of weanling rats. This suggested the above diet may be beneficial for human neonatal

carnitine dependent fatty acid transport in liver mitochondria. Results of this study also suggested current total parenteral nutrition formulas, specifically those with high linoleic fatty acid content, may adversly affect function of carnitine dependent fatty acid transport in liver mitochondria of neonates. Further study is required in these areas.

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